IMMUNOLOGICAL AND ANTITUMOUR EFFECTS OF CORYNEBACTERIUM PARVUM

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STATEMENT

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ABSTRACT

Experimental studies of the immunological and antitumour effects of the anerobic diptheroid bacterium Corynebacterium parvum are described.

C. parvum injected systemically into mice resulted in potent stimulation of the lymphoreticular system and augmented humoral immunity; however, cell mediated immunity was suppressed. The C. parvum activated macrophage was determined to be the basis for all these phenomena, exerting both stimulatory and inhibitory effects.

Using mouse tumour models both systemically and locally injected C. parvum could be manipulated to achieve therapeutic effects. The form of antitumour immunity generated varied according to the particular C. parvum tumour antigen interaction; following systemic injection, the reuslting antitumour activity was immunologically non specific and mediated directly be activated macrophages, whereas after local interaction of C. parvum with tumour antigen strong specific cell mediated antitumour immunity resulted.

Subsequent experimental studies were designed to elucidate further these stimulatory and antitumour effects of C. parvum with a view to providing a rational basis for the clinical evaluation of C. parvum against human cancer. These have comprised:-

(a) Manipulation of dose, route and time of C. parvum injection.

(b) Tissue distribution studies following injection of labelled C. parvum preparations.

(c) The role of immunity against C. parvum in its antitumour effects.

(d) The principles of combining C. parvum immunotherapy with conventional anti-cancer chemotherapy.

(e) Analyses of the active component of C. parvum.

CONTENTS

PART I INTRODUCTION AND OVERVIEW

This thesis comprises my investigations into the immunological and antitumour effects of the anaerobic diphtheroid bacterium Corynebacterium parvum (now classified as Propionobacterium acnes; see (29)[(a)].

Experimental work started in 1969; C. parvum had recently been reported to be a potent lymphoreticular stimulant and an adjuvant for both humoral and cell mediated immunity in mice, and also to be capable of inhibiting the growth of mouse experimental tumours. Similar effects were known to occur following infection of mice with living tubercule bacilli (BCG) but the attraction of C. parvum was that it was apparently active when injected as a killed bacterial suspension. The earlier C. parvum work had been with a particular strain (963 B) which was no longer available. Thus, at the outset it was necessary to screen several strains and select an active one for further investigation. A range of Corynebacteria, both aerobic and anaerobic, were obtained and assessed for both lymphoreticular stimulatory and adjuvant activity in mice. Several species were found to be active, the majority being anaerobic, but activity could not be correlated with any particular biochemical reaction (1). One strain, CN6134, showed high activity in all tests and was selected for further study.

The initial approach to understanding the mechanism(s) underlying the immunostimulatory effects of C. parvum in mice was to determine the involvement of different cell types. The status of the thymus-processed (T)

[a] numbers in parenthesis refer to published papers in Part II.

lymphocyte was assessed by the ability of lymphoid cells from C. parvum stimulated mice to respond in vitro to the T cell specific mitogen phytohaemagglutinin (PHA).

Contrary to expectation, since C. parvum had previously been described to boost in vivo T cell mediated immune competence, the PHA responsiveness of spleen cells from mice pretreated with intravenous (i.v.) C. parvum was found to be inhibited. Other recognised T cell functions, mixed lymphocyte and graft versus host reactivity, were similarly impaired; however, the deficit apparently did not extend to bone marrow derived (B) lymphocytes since the in vitro response of spleen cells to B cell mitogens was not impaired. The inhibition of T cell reactivity was restricted to spleen and peripheral blood lymphocytes there being no impaired lymph node response even after direct stimulation by locally injected C. parvum (2). The mechanism underlying the failure of C. parvum stimulated spleen T lymphocytes to respond to PHA in vitro was analysed in detail and shown not to be due to any intrinsic defect of the T lymphocyte but rather that the T lymphocyte was inhibited from responding by C. parvum activated macrophages (3).

The finding that C. parvum was capable of suppressing the in vitro expression of cell mediated immunity was intriguing in that it had been reported to have antitumour effects and, at the time, tumour immunity in experimental mice was considered to be due almost exclusively to cell mediated immunity. The next step was therefore to determine whether C. parvum could depress cell mediated immunity in vivo. This proved to be the case; mice pretreated with systemic C. parvum and sensitized with sheep red blood cells developed markedly reduced levels of delayed type hypersensitivity (DTH) against sheep cells. The effect was not due to any

inhibitory effects of antibody and showed some features reminiscent of depressed in vitro PHA reactivity, namely, it occurred after systemic rather than local injections of C. parvum and again the spleen was implicated in the suppressor mechanism (6). Since the proposed use of C. parvum was for the therapy of existing tumours (i.e. host already sensitized to tumour antigens) it was relevant to note that in vivo suppression of DTH did not occur if antigen sensitization was before, or at the same time as, C. parvum administration.

In parallel studies on the development of DTH associated bacterial infection in mice, C. parvum pretreatment suppressed the development of specific DTH associated with a subsequent infection with Salmonella enteritidis (8). It was, however, capable of protecting mice against an acute lethal injection with S. enteritidis which was in keeping with an earlier demonstration of C. parvum mediated protection against infection with a range of bacteria (7). The finding that C. parvum induced protection against S. enteritidis and yet inhibited the production of specific DTH was particularly interesting in that the elimination of such intracellular bacteria was considered to depend on the development of specific cell mediated immunity and associated DTH. Our data, however, were consistent with the idea that the early inactivation of S. enteritidis after C. parvum treatment was due to their non-specific destruction by C. parvum activated macrophages with both the liver and spleen (8).

Although systemic C. parvum had had an inhibitory influence on various T cell functions associated with cell mediated immunity, the fact that it augmented the IgG antibody response to T cell dependent antigens in mice implied that the antibody helper cell activity of T cells was not similarly impaired. The ability of C. parvum to potentiate B cell activity

was also evident from its adjuvant effect on the T cell independent antibody response to the antigen type 3 pneumococcal polysaccharides whereas another bacterial adjuvant, Bordetella pertussis, was ineffective. These and further data suggested that, as for the demonstrated T cell inhibitory effects of C. parvum, the potentiation of B cell activity was indirect and again mediated by C. parvum activated macrophages (4,5). Further work by a colleague comparing the ability of C. parvum with other conventional adjuvants to augment the antibody response to another T cell independent antigen confirmed the apparently unique lack of T cell involvement in the adjuvant effect of C. parvum[b].

My first experiments concerning the antitumour activity of C. parvum were a study of the effects of systemic C. parvum pretreatment on the in vivo growth of a variety of experimental mouse tumours; variable degrees of protection were obtained being generally greater with the more immunogenic tumours. Mice could be afforded a degree of protection against immunogenic tumours by immunization with irradiated homologous tumour cells and systemic C. parvum given before this treatment diminished the protective effect. Thus it was apparent that the somewhat anomalous immunosuppressive effects associated with C. parvum stimulation extended to immunity against tumour antigens (10).

Moving to the Trudeau Institute presented me with the opportunity to continue the C. parvum tumour studies using a tumour model known to be susceptible to immunotherapy with BCG. This was the poorly immunogenic, carcinogen induced, transplantable mouse mastocytoma P815 and it was grown as solid tumour in the footpad. The early emphasis concerned the

[b] del Guercio, P. (1972) Nature, New Biol. 238, 213.

best route of administration (11,12). C. parvum given systemically shortly after tumour establishment significantly inhibited tumour growth although no complete regressions were achieved. On the other hand a local injection of C. parvum directly into the tumour site was markedly inhibitory with a proportion of tumours regressing completely. C. parvum injected locally in the region of the tumour (i.e. stimulating the tumour draining lymph node) was also effective but only minimal tumour inhibition occurred when the injection was at a distant site.

Investigation of the mechanisms underlying the antitumour effects of C. parvum administered systemically or locally revealed striking differences between the two routes of injection. After systemic C. parvum the effects were immunologically non-specific i.e. not amplifying a specific antitumour response; they occurred in immunologically compromised (T cell deprived) mice and did not result in the generation of specific antitumour immunity. Macrophages taken from non-tumour bearing mice stimulated with systemic C. parvum inhibited the growth of tumour cells in vitro and this non-specific effect of C. parvum activated macrophages was concluded to be the major mechanism by which systemic C. parvum achieved its antitumour effect in vivo (11). Following local administration of C. parvum, macrophages were only minimally tumour inhibitory and, again in contrast with systemic C. parvum, the effects of C. parvum injected directly into the tumour lesion were entirely T cell dependent and resulted in the generation of strong, specific cell mediated immunity (12). Further characterization of the specific immunity resulting from local interaction of C. parvum with tumour antigens was achieved by substituting irradiated tumour cells for live cells thereby obviating complications associated with tumour growth (13). The immunity was cell mediated and could be generated with very low

doses of C. parvum. In keeping with the observed antitumour effects of locally injected C. parvum, the successful generation of specific immunity did not require that the injection sites of C. parvum and tumour antigen be coincident but that they did require common lymphoid drainage. Most importantly, local injection of C. parvum plus irradiated tumour cells proved capable of inhibiting the growth of established tumours thus establishing the potential of C. parvum for clinical use in a specific active immunotherapy context in situations where suitable tumour material was available.

It seemed likely that the observed differences between systemic and local C. parvum injections might be attributed to differences in in vivo distribution of the organisms. C. parvum was successfully labelled with either fluorescein or radioactive iodine without modifying its stimulatory or antitumour activities, and the relative distribution and persistence of these labelled preparations injected either locally or systemically was monitored in both normal and tumour bearing mice. The patterns obtained were readily reconciled with the characteristic profiles of lymphoreticular stimulatory and antitumour activity previously observed for the two injection routes. Also discovered was the marked resistance to degradation of the active strain of C. parvum compared with an inactive strain (19,20).

In addition to being an immunostimulant, C. parvum was an immunogen and mice treated with C. parvum, especially repeatedly, developed high levels of both cell mediated and humoral specific immunity (17). It was apparent from experiments in which 'pre-emption' of cell mediated immunity against C. parvum had reduced the efficacy of subsequent intralesional therapy that immunity against C. parvum might be implicated in its antitumour activity (12). This concept was confirmed by

the contemporary studies of others; a particular mouse fibrosarcoma was responsive to intralesional C. parvum therapy only in mice presensitized to C. parvum[c] and macrophage activation occurred as a result of contact of C. parvum with specifically sensitized T lymphocytes[d]. These data had important implications for the clinical use of C. parvum (e.g. individual patients may vary in their immune responses to C. parvum, in particular relatively anergic advanced cancer patients may respond only poorly) and prompted further studies. Unlike the mouse fibrosarcoma model, the susceptibility of mastocytoma P815 to either local or systemic C. parvum was neither dependent upon nor modified by C. parvum presensitization (14). Clearly, different tumours may be expected to vary in their susceptibility to this aspect of C. parvum mediated antitumour effects. Suspicions about the interpretation of such experiments concerning the role of C. parvum immunity were aroused by the finding of significant levels of 'natural' antibodies in many rodents and patients[e]. These most probably arose from prior infection with cross-reacting organisms and raised the question of whether a true primary response to C. parvum was ever attainable. To achieve just this we resorted to germ free mice and compared their responsiveness with their conventional counterparts. The results showed that the stimulatory effects of C. parvum could be fully established in the absence of previous sensitization (15).

[c] Tuttle, R.L. and North, R.J. (1975) J. nat. Cancer Inst. 55 1403-1409.

[d] Bomford, R. and Christie, G.H. (1975) Cell. Immunol. 17 150-155.

[e] Wolberg, G., Duncan, G.S. et al. (1977) Infect. Immun. 15 1004-1007.

There was by now considerable clinical interest in C. parvum which influenced considerably the future development of experimental animal studies. For example, it was apparent that whereas experimental mouse tumours grew rapidly allowing time for only one or few C. parvum injections, most human tumours grow slowly over a period of years and C. parvum was likely to be given repeatedly over a long period. Also it was apparent from the accumulating clinical toxicity data that the acceptable dose levels were to be considerably less than those that had been used in mice. For these reasons many of the immunological phenomena observed after single high doses of C. parvum in mice were re-evaluated using repeated, low, human equivalent doses. Both multiple, low dose systemic and local injections resulted in the development of high levels of both cell mediated and humoral immunity against C. parvum, however, the levels of non-specific stimulation and antitumour activity were significantly higher after systemic injection. A further, and most encouraging, finding was the failure to detect non-specific T cell suppression after multiple low dose regimens (17).

It was hoped that some of the clinical toxicity associated with C. parvum might be circumvented by using active fractions of the organism. To this end research aimed at determining the active component(s) of C. parvum was undertaken. Purified cell walls were prepared, analysed and compared with the whole organism for activity. Cell wall preparations retained immuno- and anti-genicity and localised and persisted in vivo in the same manner as whole cells but they lacked completely both stimulatory and antitumour activity. Considering these findings with the isolation attempts of others it was concluded that the antitumour activity was not associated with a single molecule capable of being isolated in pure form and that an association of both cell wall and non cell wall components was required (23).

Clinicians considering using immunotherapy frequently questioned the relative merits of C. parvum and BCG, in particular whether the apparent advantages of being able to use a killed preparation might be offset by reduced potency. To contribute to this debate the potentiation of specific antitumour immunity by either BCG or C. parvum was compared; larger amounts of BCG than C. parvum were required to produce similar degrees of immunity; also, stronger and more protective immunity was mediated by C. parvum than BCG (16). Encouraging results in the treatment of lung cancer patients by post-resection injection of BCG into the pleural space was reported by McKneally et al.[f] and, since the comparative mouse study had shown C. parvum apparently to be more potent than BCG, the Ludwig Lung Cancer Study Group decided to evaluate intrapleural C. parvum clinically (22). Experimental support for this trial took the form of experimental mouse studies undertaken to define the distribution of C. parvum following intrapleural injection and also the antitumour effects against lung associated tumours (21).

My final series of experimental studies with C. parvum was an analysis of the principles involved in combining immunotherapy with the more conventional cancer chemotherapy. There were already animal data indicating that such combination therapy may be a promising form of treatment, the individual antitumour effects being additive or even synergistic. A potential antagonism between the immunostimulatory effects

[f] McKneally, F.F., Maver, C.M. and Kausel, H.W. (1977) Lancet I 593.

of C. parvum and the invariably immunosuppressive chemotherapy was already apparent from my previous demonstration of the abolition of the antitumour activity of C. parvum activated macrophages by cortisone (24), and particular attention was therefore focussed on this aspect. Using a mouse fibrosarcoma model the modification of both immunologically specific and non-specific C. parvum mediated antitumour effects by drug treatment at various times was observed and related to the overall therapeutic outcome (25,26,27). The timing between the two forms of treatment was a critical factor and the principles established provided useful guidelines for the consideration and design of clinical chemo-immunotherapy trials.

Towards the end of 1979 it was considered that sufficient data had accumulated to assure the meaningful design and evaluation of trials of C. parvum against human cancer and consequently my experimental animal studies ceased. The continuing number of subsequently published experimental studies attests to the usefulness of C. parvum as a potent reagent for analysing various aspects of the immune system; an interesting development from the antitumour viewpoint has been the evidence for potentiation of Natural Killer (NK) cell activity by C. parvum[9], a property which might have been predicted from our observation of the ability of C. parvum to induce interferon (9).

[9] Ojo, E. (1979) Cell. Immunol. 45 182-187.

The potential of C. parvum as a human anti-cancer agent was widely recognised and during the late seventies and early eighties numerous clinical trials were set up. Many have now been completed and, despite the various manipulations and strategies employed, to date there has been a disappointing overall lack of significant therapeutic effects. This may reflect, at least in part, the now recognised low antigenicity of human tumours compared with many of the animal models. Also that we may have to understand further and optimize the use of immunotherapeutic agents with particular regard for the fine balance between immunostimulation and immunosuppression[h].

Two clinical studies did, however, indicate a particular role for C. parvum in the management of human cancer. Successful suppression of the rapid reaccumulation of peritoneal or pleural fluid can contribute significantly to the palliative care of patients with disseminated cancer and C. parvum injected into the peritoneal[i] or pleural[j] cavities controlled such effusions, apparently without the toxicity often associated with drug control. It was for this purpose that in 1981 C. parvum was licenced and marketed as 'Coparvax'.

(h) Davies, M. (1983) Immunol. Today 4 103-106.

(i) Webb, H.E., Oaten, S.W. and Pike, C.P. (1978) Br. Med. J. 1 338-340.

(j) Millar, J.W., Hunter, A.M. and Horne, N.W. (1980) Thorax 35 856-858.

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LYMPHO-RETICULAR STIMULATORY PROPERTIES OF *CORYNEBACTERIUM PARVUM* AND RELATED BACTERIA

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KILLED suspensions of the anaerobic diphtheroid *Corynebacterium parvum* were shown by Halpern *et al.* (1964) to possess a marked capacity for stimulating the lympho-reticular system. Treated mice showed large increases in spleen and liver weight and increased ability to clear intravenously injected carbon from the circulation. It has since been shown that *C. parvum* acts as an adjuvant (Neveu, Branellec and Biozzi, 1964; Biozzi *et al.*, 1966) and that treatment of animals with this organism can protect them from tumour-cell (Halpern *et al.*, 1966; Woodruff and Boak, 1966), protozoal (Nussenzweig, 1967) and bacterial challenge (Adlam, Broughton and Scott, 1972). Protection is also afforded against graft-versus-host disease (Howard *et al.*, 1967). In addition to these properties, *C. parvum* resembles *Bordetella pertussis*, an unrelated organism that may also act as an adjuvant (Kind, 1957) and possesses the ability to induce splenomegaly (Morse, 1965), in being able to sensitise susceptible strains of mice to endotoxin (Howard, 1968) and to histamine (Adlam *et al.*).

The work of Prévot and Tran Van Phi (1964), who showed that several diphtheroid species other than *C. parvum* stimulate the reticulo-endothelial system to take up injected carbon more rapidly, suggested that the properties possessed by *C. parvum* might be common to several related species. This possibility was also indicated by the work of Farber and Smith (1969), who showed that a strain of *C. acnes* possessed reticulo-endothelial stimulatory properties similar to *C. parvum.* In addition, both *C. rubrum* (Katsh, 1960) and *C. avidum* (Giambanco, Armani and Falcone, 1966), like *C. parvum* (Neveu *et al.*), may be satisfactorily substituted for the mycobacterial component of Freund's complete adjuvant.

In the present work, several species of corynebacteria were quantitatively

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tested for lympho-reticular stimulatory activity and an attempt has been made to relate their biochemical reactions to their stimulatory ability. The following parameters of lympho-reticular stimulation were examined: increase of spleen and liver weight; carbon clearance; adjuvant effect; and sensitisation of mice to histamine.

METHODS

Bacterial strains. Strains obtained from various sources (table I) were maintained as freeze-dried seed cultures in the Wellcome Culture Collection.

Media. Bacterial growth media were Wellcome Reagents, unless otherwise indicated.

TABLE I

Culture collection numbers of strains of corynebacteria

Strains	Culture collection numbers				
	NCTC*	ATCC†	Pasteur Institute	Wellcome	Sources‡ of strains
C. acnes	...	11828	...	CN 6280	(1)
C. acnes	...	...	...	CN 6276	(1)
C. anaerobium	...	...	4685	CN 6134	(2)
C. betae	...	...	...	CN 4229	(3)
C. diphtheriae§	7429	296	...	CN 2000	(3)
C. diphtheroides	...	...	2764	CN 6295	(1)
C. equi	...	...	...	CN 4686	(3)
C. granulosum	...	...	3024-B	CN 6292	(1)
C. laevaniformans	...	15923	...	CN 6366	(4)
C. liquefaciens	...	...	3044-B	CN 6290	(1)
C. lymphophilum	...	...	...	CN 6294	(1)
C. parvum (*a*)	10387	11829	...	CN 5888	NCTC
(*b*)	10387	11829	...	CN 6293	(1)
(*c*)	10387	11829	...	CN 6156	(5)
C. parvum	10390	12930	643-C	CN 5936	NCTC
C. pyogenes	...	...	637-B	CN 6291	(1)

* National Collection of Type Cultures, Central Public Health Laboratories, Colindale Avenue, London, N.W.9.

† American Type Culture Collection, Rockville, Md, USA.

‡ Sources of strains: (1) Professor R. F. Smith, Department of Microbiology, Temple University School of Pharmacy, Philadelphia, Pa., USA. (2) Professor M. Raynaud, Institut Pasteur, Annexe de Garches, Garches, 92 Hauts-de-Seine, France. (3) Wellcome Culture Collection. (4) Dr J. Miranda, Wellcome Research Laboratories. (5) Dr K. C. Milner, Department of Health, Education and Welfare, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana, USA.

§ Park Williams no. 8 strain.

Growth of bacteria. Strains of *C. acnes*, *C. anaerobium*, *C. diphtheroides*, *C. granulosum*, *C. liquefaciens*, *C. lymphophilum*, *C. parvum* and *C. pyogenes* were grown anaerobically. Organisms from freeze-dried vials were sub-cultured in Robertson's meat broth (20 ml) containing 5 per cent. horse serum and 1 per cent. glucose. After static incubation for 7–10 days at 37°C organisms were transferred to 1 per cent. glucose broth (100 ml) and re-incubated for 7–10 days. For bulk growth this culture was used to seed 2-l bottles containing glucose broth which were then statically incubated at 37°C for 7–10 days.

C. betae and *C. laevaniformans* strains were grown aerobically on nutrient agar slopes for 48 hr at 20°C before subculture into 2-l bottles of brain heart infusion broth (Oxoid Ltd) and incubation with shaking at 20°C for 3 days. The *C. equi* strain was subcultured in brain heart infusion broth (20 ml) and incubated at 37°C for 5–7 days. For bulk growth

this culture was used to seed 2-l bottles containing brain heart infusion broth which were then incubated with shaking at 37°C for 5–7 days. The *C. diphtheriae* strain was grown in 100-ml bottles of papain digest of beef (Ramon, Pochon and Amoureux, 1941) supplemented with ferric citrate (10 μg per ml) to abolish toxin formation (Edwards and Seamer, 1960). For bulk growth this culture was used to seed 300-ml bottles of media which were shaken at 37°C for 48 hr.

Killing and harvesting bacteria. Only killed organisms were used for animal inoculation. All cultures were killed by addition of formalin to 0·5 per cent.; after standing overnight, they were centrifuged and washed three times in pyrogen-free physiological saline before being suspended in the same medium with 0·01 per cent. thiomersalate as preservative.

Dry weight of bacteria. Organisms were washed three times in filtered glass-distilled water (Sartorius filter, 0·2 μm pore size; V. A. Howe and Co. Ltd, 88 Peterborough Road, London, S.W.6). Samples (1·0 ml) of known optical density were dispensed in triplicate into weighed bottles and dried at 60°C to constant weight.

Carbohydrate fermentation. A dense suspension of organisms was inoculated into 1 per cent. peptone sugars and the formation of acid noted over a 3-wk period. Incubation was at 37°C except in the case of *C. betae* and *C. laevaniformans* strains which were incubated at 20°C. For anaerobic strains, 0·2 per cent. nutrient agar was added to the peptone medium.

Gelatin liquefaction. Tubes of nutrient gelatin were stab-inoculated and examined periodically over 3 weeks by placing in the refrigerator at 4°C for 30 min.

Catalase production. A dense suspension of organisms was mixed on a microscope slide with a drop of freshly prepared hydrogen peroxide (5 per cent.). The production of effervescence was noted.

Bromocresol purple milk. Tubes of milk medium containing indicator (Cowan and Steel, 1965, p. 160) were inoculated with organisms and examined daily for 14 days for acid and clot production.

Indole production. This was tested for by the method of Cowan and Steel (p. 158) using Ehrlich's reagent as indicator.

Nitrate reduction. This was tested for by the method of Cowan and Steel (p. 161). Inoculated broth was incubated for 5 days and the reaction elicited by the addition of sulphanilic acid and α-naphthylamine.

Measurement of lympho-reticular stimulation

Increase in organ size. Groups of ten female W-Swiss mice (16–20 g, S. Schofield and Co. Ltd, Intake Head, Delph, Oldham, Lancs) received a single intravenous injection of organisms in varying doses. Animals were killed by cervical dislocation at varying days after treatment and their spleens, livers and lungs weighed immediately; body weights were also measured. Mean values for organ weights were corrected for body weights so that they could be expressed as mg per 20 g body weight. Control animals that had received thiomersalate saline were included in each experiment.

Carbon clearance. Groups of eight female CBA/T6 mice (16–20 g, reared at the Wellcome Laboratories) received a single intravenous injection of organisms (1·4 mg dry weight). At intervals thereafter animals were assayed for their ability to clear intravenously injected colloidal carbon from the circulation. Phagocytic indices (K values) were calculated by the method of Biozzi, Benacerraf and Halpern (1953). Control animals injected with thiomersalate saline were inoculated in each experiment.

Adjuvant effect. Groups of ten male CBA *p* mice (16–20 g, reared at the Wellcome Laboratories) received a single intravenous injection of organisms (1·4 mg dry weight). Four days later the animals were given intravenous injections of 0·25 ml of a 10 per cent. suspension of washed sheep red cells (Wellcome Reagents, Ltd) in saline (5×10^8 cells) and were afterwards bled (0·05 ml per mouse) at intervals. Sera pooled from ten animals were assayed for their haemagglutination titre by microtitration in conical plastic wells (Linbro Chemical Co. Inc., 681 Dixwell Avenue, Newhaven, Conn. 06511, USA). Serial two-fold dilutions of sera in physiological saline were added in 0·05-ml volumes to an equal volume

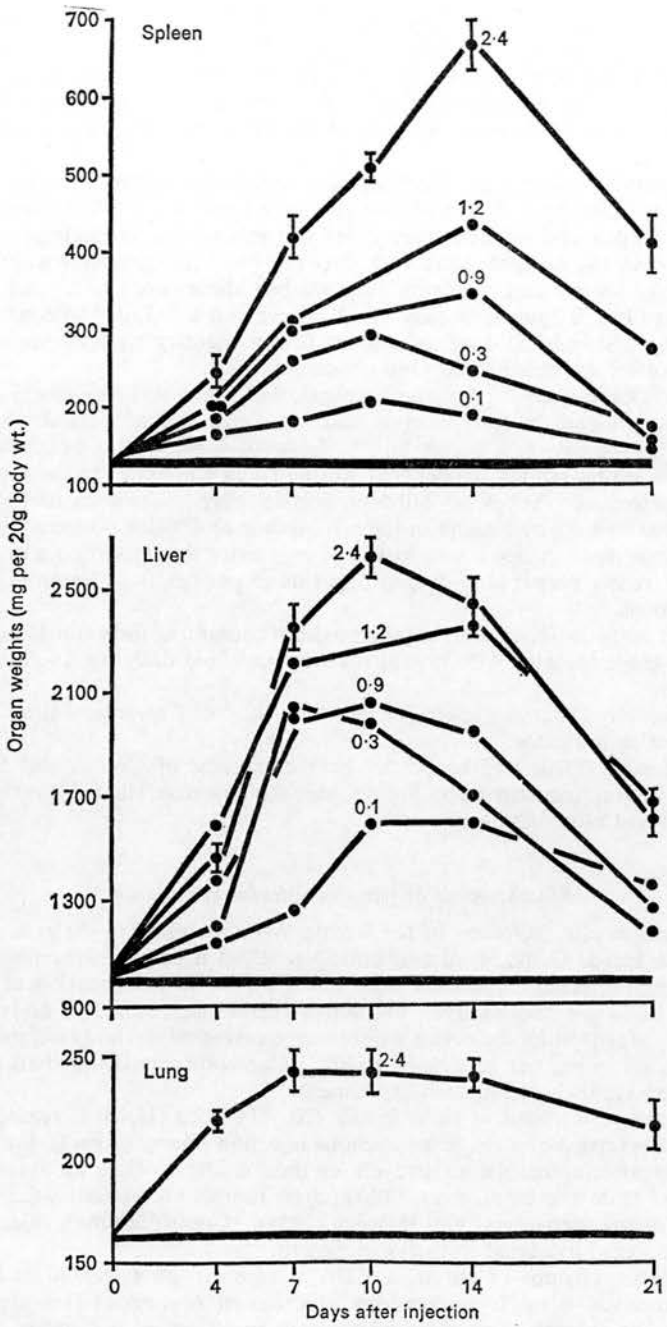

FIG. 1.—The weights of internal organs of mice at different times after injection of varying doses of *Corynebacterium anaerobium*.

Groups of ten mice received killed organisms intravenously; numbers identifying the curves are dry weights (mg) of organisms injected. Animals were then killed at intervals and organ and total body weights determined. Organ weights were corrected for body weights; standard errors are shown for the animals that received 2·4 mg of bacteria. The solid bar indicates organ weights and standard errors for all control animals studied during the course of the experiments.

of washed sheep red cells (1 per cent.); duplicate titrations were made. After incubation (1 hr at 37°C, then overnight at 4°C), titres were recorded as the reciprocal dilution of the last well showing agglutination.

Histamine-sensitising effect. Groups of ten female W-Swiss mice (16–20 g, Bromfield Ltd, 81 Windsor Avenue, Newton Abbot) received a single intravenous injection of organisms at varying doses. Each animal was challenged 7 days later with an intraperitoneal injection of histamine phosphate (1 mg histamine base; B.D.H. Ltd, Poole, Dorset). Deaths were recorded over a 24-hr period. Control animals that had received intravenous thiomersalate saline were included in each experiment and were challenged with 5 mg histamine base per mouse.

RESULTS

Increase in organ size. After a single intravenous injection of an active strain, the spleen, liver and lung weights were markedly increased. As an example, positive responses to varying doses of *C. anaerobium* are shown in fig. 1. After an injection of 2·4 mg (dry weight) of this organism, a five-fold increase in spleen weight and a three-fold increase in liver weight occurred. Maximum weights of spleen and liver were reached 10–14 days after treatment. A small but significant increase in lung weight was observed between 7 and 14 days after injection.

TABLE II

Spleen, liver and lung weights of mice after the injection of different strains of corynebacteria

Strain CN number	Species	Spleen index* at 14 days	Liver index* at 10 days	Lung index* at 10 days
6134	*C. anaerobium*	5·09±0·26	2·49±0·09	1·45±0·08
6280	*C. acnes*	5·06±0·35	2·55±0·09	1·53±0·10
6276	*C. acnes*	4·84±0·19	2·41±0·11	1·36±0·06
6295	*C. diphtheroides*	4·78±0·28	2·49±0·13	1·38±0·05
6291	*C. pyogenes*	4·69±0·20	2·36±0·04	1·32±0·03
6290	*C. liquefaciens*	4·67±0·17	2·46±0·10	1·40±0·09
6294	*C. lymphophilum*	4·58±0·32	2·46±0·10	1·71±0·08
6293†	*C. parvum*	4·10±0·36	1·89±0·08	1·12±0·04
5936	*C. parvum*	3·94±0·33	2·17±0·14	1·27±0·06
6292	*C. granulosum*	3·40±0·39	2·29±0·11	1·28±0·05
4686	*C. equi*	2·73±0·21	1·89±0·09	1·01±0·02
6156†	*C. parvum*	2·52±0·19	1·41±0·09	1·26±0·12
6366	*C. laevaniformans*	1·83±0·16	1·04±0·03	1·16±0·04
5888†	*C. parvum*	1·37±0·09	1·01±0·03	1·24±0·02
2000	*C. diphtheriae*	1·21±0·10	1·00±0·02	1·17±0·03
4229	*C. betae*	1·12±0·05	1·10±0·01	1·10±0·04

* Groups of ten mice received a single intravenous injection of killed organisms (2·4 mg dry wt). Animals were killed and organs weighed after 10 or 14 days. Figures in the body of the table are organ indices with standard errors. Organ index $= \frac{\text{mean organ weights of treated animals}}{\text{mean organ weights of control animals}}$.

† The same strain obtained from three different sources.

The extent of increase in organ weights produced by all organisms tested is shown in table II. For ease of comparison, strains are arranged in approximate order of maximum activity. Organ-weight increase is expressed as an index by comparison with control values. The kinetics of organ-weight increase were similar for all active strains, and a marked increase in spleen weight was always accompanied by an increase in liver and lung weights. Although

greatest activity was shown by anaerobic strains, not all were active and not all aerobic strains were inactive since *C. equi* showed moderate activity. A culture of *C. parvum* (CN 5888) obtained from the National Collection of Type Cultures was inactive, although two cultures of the same strain (CN 6293 and 6156) obtained from American laboratories possessed some activity.

Carbon clearance. Experiments with *C. anaerobium* showed that maximum carbon clearance was produced 4 days after bacterial inoculation, the kinetics of the response (fig. 2) being similar over a wide range of dose of organisms.

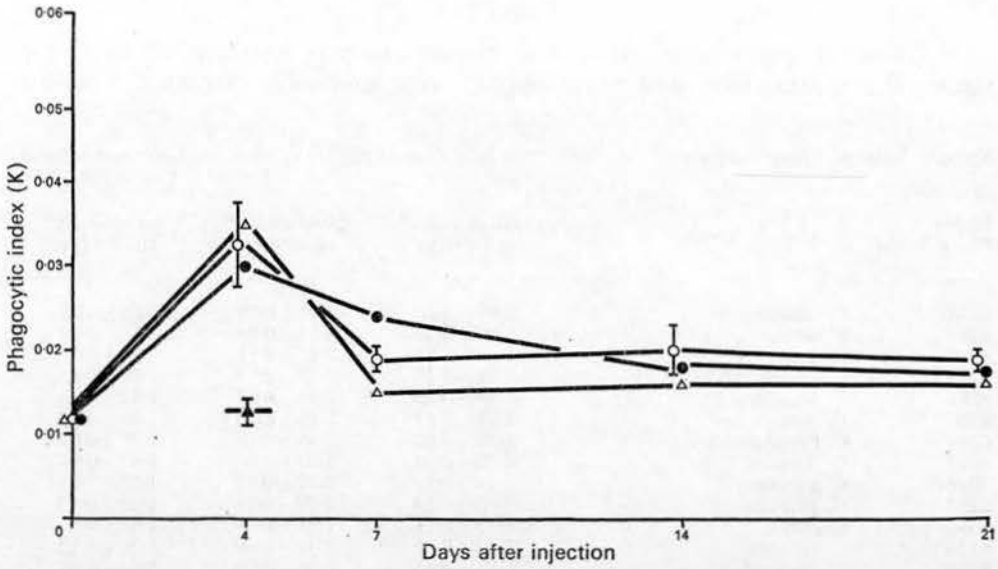

FIG. 2.—Enhancement of carbon clearance from the circulation by *Corynebacterium anaerobium*. Groups of eight mice received different doses of killed organisms intravenously. Carbon clearance indices (K values) were determined at intervals subsequently. Standard errors of values are shown for the animals that received 1·2 mg dry weight of organisms. The single control point shows the mean value and standard error of control animals throughout the experiment. K values: ○——○ after 1·2 mg; ●——● after 0·9 mg; △——△ after 0·3 mg of organisms; —▲— in controls.

Fig. 3 shows the results of carbon-clearance assays for eight of the strains of corynebacteria. K values of phagocytosis are shown in the form of a histogram with high carbon clearance strains on the left. Whereas *C. anaerobium* and *C. acnes* (CN 6134 and 6276) were most active and *C. granulosum* and *C. lymphophilum* (CN 6292 and 6294) also showed activity, *C. parvum* and *C. diphtheriae* (CN 5936 and 2000) were doubtfully active. *C. betae* and another *C. parvum* strain (CN 4229 and 5888) were inactive.

Adjuvant effect. Experiments with *C. anaerobium* showed that peak adjuvant activity to sheep red-cells was produced by injecting the organisms 4 days before the red cells. Table III shows the results of an experiment in which nine of the strains of corynebacteria were used in this way. Results have been expressed as $\log_2$ haemagglutination titres, strains being arranged in the order of highest titre attained. Strains CN 6294, CN 5936, CN 6290, CN 6292, CN 6134, CN 6295 and CN 6276 showed marked activity that reached a peak

6–8 days after injection of sheep cells. Strains CN 5888 and CN 2000 were inactive.

Histamine sensitisation. Table IV shows the combined results of two experiments in which strains were tested for their ability to sensitise mice to histamine. Results have again been grouped in order of activity of the strains.

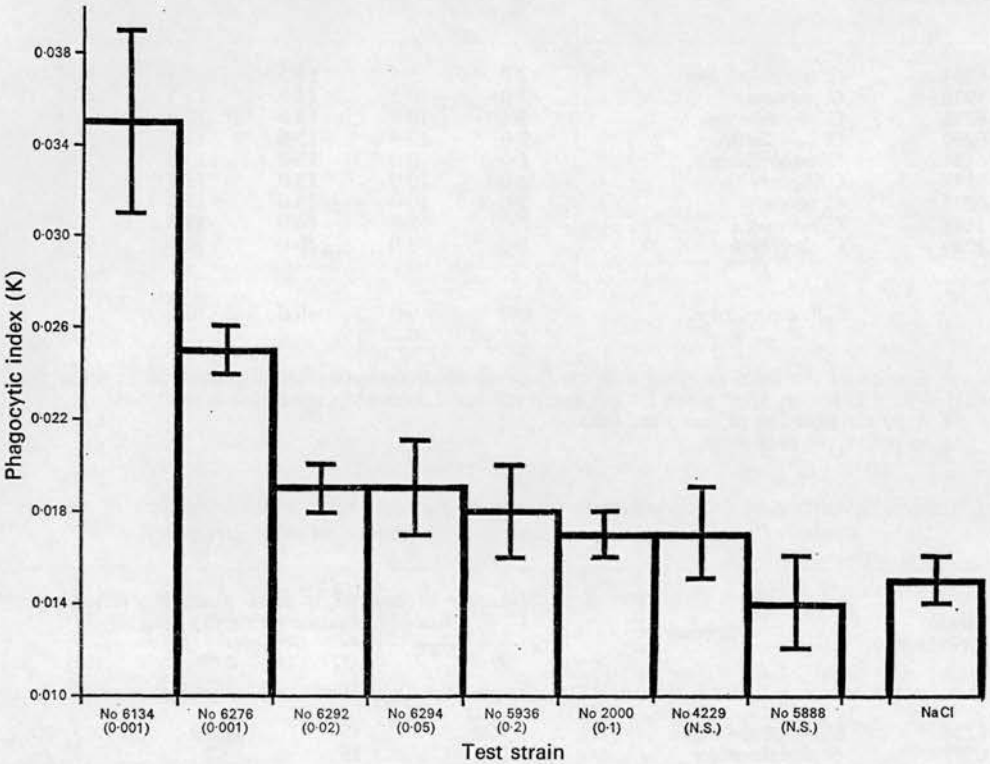

FIG. 3.—The ability of different strains of corynebacteria to enhance carbon clearance from the circulation. Groups of eight mice received a single intravenous injection of killed organisms (1·4 mg dry weight). Four days later carbon clearance assays were performed and the phagocytic indices (K values) so obtained are indicated by the heights of columns in the histogram, with standard errors. The Wellcome Collection (CN) number of each strain is shown at the foot of the relevant column (see table I for identity of strains). NaCl = K value in control mice receiving saline. The numbers in parentheses are the probabilities (P) of significance of differences between test results and controls. N.S. = Not significant.

In terms of dry weight of organisms required to sensitise, *C. lymphophilum* possessed the highest activity and strains of *C. acnes*, *C. anaerobium*, *C. diphtheroides*, *C. granulosum* and *C. liquefaciens* were quantitatively similar in activity. *C. pyogenes* and *C. parvum* (CN 5936) were low in activity and the remaining strains were inactive or caused a low-grade irregular sensitisation unrelated to dose of organisms injected.

Taxonomy. The results of the biochemical tests are shown in table V. All corynebacteria tested fermented glucose and produced catalase. In addition, all strains with the exception of *C. equi* fermented galactose, mannose and fructose. *C. equi* was exceptional, not only in its inability to ferment any

TABLE III

*Adjuvant activity of corynebacteria: haemagglutinin titres in mice given injections of sheep red cells after pre-treatment with different strains of corynebacteria**

Strain CN number	Species	Log_2 haemagglutination titre on day†				
		2	4	6	8	10
6294	*C. lymphophilum*	2·0	9·5	13·5	**15·5**‡	11·5
5936	*C. parvum*	2·0	10·5	**14·5**	13·5	13·5
6292	*C. granulosum*	3·5	10·0	**14·0**	12·5	11·5
6290	*C. liquefaciens*	2·0	10·5	**13·5**	13·5	13·5
6134	*C. anaerobium*	2·0	9·0	**13·0**	13·0	13·0
6295	*C. diphtheroides*	2·0	10·0	**13·0**	13·0	12·0
6276	*C. acnes*	2·0	10·0	**13·0**	13·0	12·0
5888	*C. parvum*	2·0	8·5	10·0	**11·5**	11·5
2000	*C. diphtheriae*	2·0	8·0	10·0	10·0	**10·5**
	Saline control	2·0	9·0	**10·0**	10·0	10·0

* Groups of ten mice received a single intravenous injection of killed organisms (1·4 mg dry wt); 4 days later they were given 5×10^8 sheep red cells; then they were bled at intervals.
† After the injection of sheep red cells.
‡ In bold type, peak titre.

TABLE IV

*Histamine sensitisation by corynebacteria: death rates of mice given injections of various strains of corynebacteria and subsequently challenged with histamine**

Strain CN number	Species	Number of deaths† in mice given the following doses of organisms (mg dry weight)			
		0·80	0·27	0·09	0·03
6294	*C. lymphophilum*	20	19	18/19	17
6295	*C. diphtheroides*	18/19	16	12	9
6134	*C. anaerobium*	16/19	19	8	10
6276	*C. acnes*	19	17	10	4
6290	*C. liquefaciens*	19	18	9	3
6280	*C. acnes*	19	14	12	3
6292	*C. granulosum*	19	13	8	8
6291	*C. pyogenes*	17	11	5	1
5936	*C. parvum*	17	10	1	2
4686	*C. equi*	14	9	3	3
5888‡	*C. parvum*	7/19	5	6	6
6293‡	*C. parvum*	4/16	8	6	9/19
2000	*C. diphtheriae*	5	6	5	7
6366	*C. laevaniformans*	4	3	2	2
6156‡	*C. parvum*	3	3	1	2
4229	*C. betae*	0	1	5/19	5
	Saline control	3/40	...	...	...

* Groups of mice received varying doses of organisms intravenously. Seven days later, animals were challenged with 1 mg histamine base intraperitoneally (saline-control animals received 5 mg base). Deaths were recorded 24 hr after challenge.
† Number of deaths per 20 mice, unless otherwise indicated.
‡ The same strain obtained from three different sources.

TABLE V

Biochemical reactions of the strains of corynebacteria

Organism	Wellcome number (CN)	† Fermentation of: Glucose	Lactose	Sucrose	Maltose	Mannitol	Starch	Galactose	Trehalose	Mannose	Fructose	Dulcitol	Dextrin	Sorbitol	Rhamnose	Raffinose	Arabinose	Salicin	Inositol	Inulin	Xylose	Adonitol	Glycerol	NO_3 reduction	BCP milk*	Indole production	Gelatin liquefaction	Catalase production
Anaerobic species																												
C. acnes	6276	+	−	±	+	+	−	+	+	+	+	−	+	+	−	−	+	−	+	−	−	±	+	+	+	+	+	+
C. acnes	6280	+	−	−	±	+	−	+	+	+	+	−	±	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+
C. anaerobium	6134	+	−	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+
C. granulosum	6292	+	−	−	−	−	−	+	+	+	+	−	−	+	−	−	−	−	+	±	−	+	+	+	±	+	+	+
C. liquefaciens	6290	+	−	−	−	+	−	+	+	+	+	−	+	+	−	−	±	−	−	+	−	−	+	+	+	+	+	+
C. lymphophilum	6294	+	−	−	+	−	+	+	−	+	+	−	+	+	+	+	−	−	+	−	−	−	+	+	+	+	+	+
C. parvum	5936	+	−	−	−	±	−	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
C. parvum‡ (*a*)	5888	+	±	+	+	−	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	±	+	+	+	+	+
(*b*)	6293	+	−	+	+	−	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	±	+	+	+	+	+
(*c*)	6156	+	−	+	+	−	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	±	+	+	+	+	+
C. pyogenes	6291	+	−	+	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+
Aerobic species																												
C. betae	4229	±	+	+	−	±	−	+	+	+	+	−	−	−	+	−	±	+	±	−	+	±	+	−	+	−	−	+
C. diphtheriae	2000	+	−	−	±	−	+	+	−	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+
C. equi	4686	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+
C. laevaniformans	6366	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	±	+	+	+	+	+	−	−	+	−	+	+

* Bromocresol purple milk. † + = Positive, ± = variable, and — = negative reaction. ‡ See table I.

of the sugars tested (except for weak glucose fermentation), but also in its production of a bright pink pigment. *C. betae* and *C. laevaniformans* were the only strains possessing bright yellow pigmentation.

As a group, the anaerobic strains showed similar biochemical reactions. Particularly close patterns of reaction were observed amongst the two *C. acnes* strains, *C. anaerobium*, *C. liquefaciens*, *C. pyogenes* and the *C. parvum* strains. All three cultures of *C. parvum* NCTC 10387 (CN 5888, CN 6293 and CN 6156), although obtained from different sources, gave very similar reactions.

TABLE VI

Publications by other authors describing the properties of the same strains of corynebacteria as those used in this study and their conclusions about taxonomy

Species	Wellcome number (CN)	Reference	Authors' conclusions and recommendations
C. acnes	6280	Smith and Bodily (1968)*; Zierdt, Webster and Rude (1968); Reid and Joya (1969)†	Used as reference *C. acnes*
C. diphtheriae	2000	Lampidis and Barksdale (1971)	" *Mitis* " strain
C. diphtheroides	6295	Zierdt *et al.*	Transfer to *C. acnes*
C. granulosum	6292	Zierdt *et al.*; Moss *et al.* (1969)	Transfer to *C. acnes*
C. laevaniformans	6366	Dias and Bhat (1962, 1964)	New species producing laevan
C. liquefaciens	6290	Zierdt *et al.*; Moss *et al.*	Transfer to *C. acnes*
C. parvum	5888	Werner and Mann (1968)	Mixed culture of *C. parvum* and *C. acnes*
		Reid and Joya	Transfer to *C. acnes*
C. parvum	5936	Werner and Mann	Transfer to *C. acnes*
C. pyogenes	6291	Moore, Holdeman and Cummins (1968)	Not a typical *C. pyogenes*
		Zierdt *et al.*; Moss *et al.*	Transfer to *C. acnes*

* Strain termed *Propionibacterium acnes* by authors.
† Strain termed *C. liquefaciens* by authors.

DISCUSSION

The taxonomy of the corynebacteria remains a matter of considerable controversy. Several workers are of the opinion that some of the anaerobic species described by Prévot (1966), e.g., *C. anaerobium*, *C. avidum*, *C. diphtheroides*, *C. granulosum*, *C. liquefaciens*, *C. lymphophilum* and *C. parvum* should be re-classified as *C. acnes* (Zierdt, Webster and Rude, 1968; Moss *et al.*, 1969; Reid and Joya, 1969). Others disagree with this view (Moore, Holdeman and Cummins, 1968), and some consider that the *C. acnes* species contains two distinct groups of organisms (Brzin, 1964; Voss, 1970). To add further confusion, there seems little doubt that the type species *C. diphtheriae* differs markedly from these anaerobic species (Barksdale, 1970), and that *C. pyogenes* and the plant diphtheroids do not resemble *C. diphtheriae* (Cummins, 1962; Barksdale). Table VI gives literature references to work in which the

strains of corynebacteria studied were identical with those used in the present work.

The question arises as to how many of these species possess the lympho-reticular stimulatory activity described for a strain of *C. parvum* by Halpern *et al.* (1964). In addition it may be asked how far other stimulatory strains possess the attributes of *C. parvum*. The present work has confirmed that several species of corynebacteria besides *C. parvum* possess lympho-reticular stimulating properties. The increase in lymphoid organ size produced by active strains was similar to that previously described for *C. parvum* (Halpern *et al.*, 1964) and the uniformity of kinetics of organ-weight increase produced by active strains suggests that these organisms contain similar if not identical stimulating material. Organisms that were able to act as adjuvants to sheep red cell agglutinin formation also increased lymphoid organ size and possessed the ability to sensitise mice of the strain used to histamine. Conversely corynebacteria lacking adjuvant activity also failed to cause histamine sensitisation or to produce marked increases in lymphoid organ size.

In the present study, enhancement of carbon clearance, even by strains highly active in organ weight-increasing ability, was lower than that originally observed with a strain of *C. parvum* (Halpern *et al.*, 1964). However, different strains of mice have been shown to vary in response to *C. parvum* treatment in this respect. A large increase in organ size was not always paralleled by concomitant enhancement of carbon clearance (Stiffel *et al.*, 1970). Nevertheless, in the present work, only those strains that increased organ size and possessed adjuvant and histamine-sensitising effects also caused a marked increase in carbon clearance.

The biochemical reactions exhibited by the strains confirm the close relationships amongst the anaerobic corynebacteria noted by other workers. As found by Zierdt *et al.* and Reid and Joya, gas production and gelatin liquefaction proved to be unreliable criteria for distinguishing between species. For example, the *C. anaerobium* strain when grown in bulk occasionally produced gas from glucose broth, which would place it in the *C. parvum* group of organisms according to Prévot. Also *C. parvum* strain CN 6156 did not liquefy gelatin, but is surely the same species as strains CN 5888 and CN 6293.

It must be concluded that the ability to stimulate the lympho-reticular system cannot, at this stage, be linked with any specific biochemical test. This is shown particularly well in the case of *C. equi* which possessed moderate capacity to increase spleen and liver weight and to sensitise to histamine, but was exceptional in its lack of biochemical reactivity. In addition, the three strains of *C. parvum* (NCTC 10387) had almost identical biochemical reactions but differed markedly in ability to increase organ weight.

The strain of *C. pyogenes* used in these studies is probably atypical since it produces catalase and indole and reduces nitrate (Moore *et al.*; Roberts, 1968). Its cell-wall amino acid composition is similar to that of other *C. pyogenes* strains but different from that of *C. acnes* (Cummins and Harris, 1956). This would suggest that the ability to stimulate the lympho-reticular system does not depend on the qualitative nature of these cell-wall amino acids. In view

of the work of Moss *et al.*, the cell-wall fatty acids may be a more important guide to the possession of stimulatory activity by any individual strain, but this remains a subject for further study.

SUMMARY

Several aerobic and anaerobic species of corynebacteria were examined for their ability to stimulate the lympho-reticular system of mice. Whereas active strains produced marked increases in spleen, liver and lung weight, possessed adjuvant activity, and were capable of sensitising mice to histamine, inactive strains possessed none of these attributes. Enhancement of carbon clearance was observed only in strains that possessed the other stimulatory properties but was not as great as previously observed by other workers for *Corynebacterium parvum*. The majority of active strains were anaerobic and possessed quantitatively similar stimulating activities. No correlation was observed between biochemical reactions of strains and lympho-reticular stimulation.

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Biological Effects of the Adjuvant *Corynebacterium Parvum*

I. Inhibition of PHA, Mixed Lymphocyte and GVH Reactivity

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Spleen and peripheral blood lymphocytes from mice injected 7 days previously with bacterial adjuvant *Corynebacterium parvum* were found to be refractory or markedly unresponsive to PHA stimulation *in vitro*. Other recognised T-cell functions—mixed lymphocyte and graft-versus-host reactivity were similarly reduced. These results are interpreted as showing that the T-lymphocyte is affected by *C. parvum*. Studies with pokeweed mitogen (PWM) gave no evidence for a similar effect on the B lymphocyte.

INTRODUCTION

Injection of killed suspensions of *Corynebacterium parvum* results in massive proliferation of the cells of the lymphoreticular system (1) and increased antibody production to subsequently injected antigens (2). Other effects of this organism–antitumour activity (3), protection against bacterial infection (4), raising the threshold for tolerance induction (5) and induction of autoimmunity (6) are all in keeping with its role as an adjuvant at both cellular and humoral levels. Augmentation of cell-mediated immunity is further reflected in its ability to induce delayed hypersensitivity to unrelated antigens (2).

The present experiments were undertaken as part of a series designed to elucidate the mechanism whereby *C. parvum* might be effective in some of the biological systems described. The approach has been to examine the phytohaemagglutinin (PHA) responsiveness of lymphoid cells from mice treated with *C. parvum*. The PHA responsiveness is restricted almost exclusively to thymus-derived cells—T-lymphocytes (7) and has been correlated with cell-mediated immunocompetence.

There have been numerous reports that lymphocytes from various immunologically deficient states have shown impairment in their *in vitro* transformation by PHA (8–10) but, thus far, this study would appear to constitute the first examination of PHA responsiveness of lymphocytes from animals with "elevated" immune status.

MATERIALS AND METHODS

Animals. Adult mice of inbred strain CBA-*p* were used throughout the PHA studies.

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Bacteria. Corynebacterium parvum suspension (Batch No. EZ174, 7 mg/ml) was prepared by the Wellcome Research Laboratories, Beckenham, Kent, England. A formalin-killed suspension of *C. diphtheriae* (Park-Williams Strain No. 8) was kindly provided by Dr. C. Adlam. Standard doses of 0.2 ml were injected intravenously 7 days prior to preparation of cell suspensions.

Cell suspensions. Spleen and lymph nodes (pooled axillary, brachial, inguinal and mesenteric) were homogenised in Eagles Minimum Essential Medium (MEM) using ground glass homogenisers. The resulting cell suspensions were filtered through both coarse and fine stainless steel sieves and washed in MEM.

Lymphocyte suspensions from blood were prepared as follows. Mice were asphyxiated with carbon dioxide, the thoracic cavity opened and 0.25 ml of heparinised saline (100 units/ml) injected into the left ventricle. The heart and major vessels were incised, the blood of six or seven mice was pooled and the red cells sedimented by addition of 0.75 vol of 3.5% dextran in saline. After incubation at 37°C for 30 min, the plasma/dextran–leucocyte layer was removed and the cells centrifuged at 4°C and washed twice with cold MEM.

Response to PHA in vitro. All lymphoid cell suspensions were adjusted to a final concentration of 10^6/ml in MEM containing 10% calf serum (Flow Laboratories). One-milliliter aliquots were dispensed into round bottomed Sterelin tubes and 0.1 ml of the appropriate concentration of PHA (Purified Phytohaemagglutinin, Wellcome Reagents Ltd., London) diluted in MEM was added. Cultures in triplicate were incubated at 37°C in vacuum desicators containing a final gas mixture of 10% oxygen, 87.5% nitrogen and 2.5% carbon dioxide.

After 48 hr, 0.25 μCi of [^{14}C]-thymidine (^{14}C-T) in 0.1 ml Hanks' Balanced Salt Solution was added to each culture which was incubated for a further 24 hr. The amount of radiolabel incorporated into the acid precipitable material was assessed by liquid scintillation counting as described by Ivanyi *et al.* (11) except that Whatman glass fibre papers (2.5 cm GF/B) were substituted. The results are expressed in counts per minute or as a ratio (stimulation index) : cpm in experimental cultures/cpm in control cultures.

Response to pokeweed mitogen in vitro. Aliquots (0.1 ml) of pokeweed mitogen (Grand Island Biological Co.) reconstituted in 5 ml sterile saline were added to cultures and uptake of ^{14}C-T assessed as for PHA.

Mixed lymphocyte reaction (MLR). Spleen cells (10^6) from normal CBA-*p* mice, or mice that had been injected 7 days previously with 0.2 ml *C. parvum* intravenously, were mixed with an equal number of inactivated syngeneic or allogeneic (DBA/2) normal spleen cells. Inactivated target cells were taken from mice which had received 1000 rads whole body irradiation from a ^{60}Co source immediately prior to use. These cells showed no increased DNA synthesis on stimulation with an optimal PHA dose, such controls being included in each experiment. Culture conditions were as described for PHA stimulation. The ^{14}C-T was added after 72 hr and the cultures terminated at 96 hr for assay of isotope incorporation.

Graft-versus-host reactivity. The graft-versus-host (GVH) action of lymphoid cells from parental (C57BL) mice was assayed by their ability to cause splenomegaly when injected into (CBA-T6T6/C57BL)F_1 hybrid mice. Spleens were assayed 9 days after injection of cells.

T-cell-deprived mice. Six- to eight-week-old mice were thymectomised and 6 days later given 900 rads whole body irradiation. The next day they received 5×10^6 syngeneic bone marrow cells intravenously and spleen preparations were made 5–6 weeks after reconstitution.

EXPERIMENTAL

Immunostimulatory, adjuvant and antitumour effects of C. parvum. The strain of *C. parvum* used has been demonstrated to cause splenomegaly, hepatomegaly, increased carbon clearance and to show adjuvant activity when injected intravenously 7 days before sheep erythrocytes (12). Pretreatment with *C. parvum* 7 days prior to challenge with syngeneic tumour cells also affords the mice considerable protection, in terms of survival (Scott, unpublished results).

Effect of pretreatment with C. parvum on the PHA responsiveness of various lymphoid populations. Spleen, lymph node (pooled) and peripheral blood cells from mice which had received 0.2 ml of *C. parvum* suspension (7 mg/ml) intravenously 7 days previously, were tested for their ability to respond to various concentrations of PHA *in vitro*. Popliteal lymph node cells from mice which had received 0.025 ml *C. parvum* suspension subcutaneously in both hind footpads were similarly tested after 7 days.

a. Spleen cells (Fig. 1A). Cells from normal mice responded to PHA in terms of greatly increased DNA synthesis. The optimal PHA dose for 1×10^6 cells was 0.001 ml PHA/ml medium, both higher and lower doses resulting in reduced stimulation.

Cells from *C. parvum*-stimulated mice were found to be refractory to all doses of PHA. The DNA synthesis was decreased compared with unstimulated cells only with the highest dose, suggesting that PHA was here having a cytotoxic effect. The background incorporation of ^{14}C-T into *C. parvum*-stimulated cells in the absence of PHA was not significantly different from that of normal unstimulated cells.

b. Lymph node cells (Fig. 1B). Cells from both normal and *C. parvum*-stimulated mice responded to PHA, the dose responses of both cell populations being similar. Again, the background ^{14}C-T incorporation of normal and *C. parvum*-treated cells was not different.

c. Draining (popliteal) lymph node cells (Fig. 1C). The background ^{14}C-T incorporation into *C. parvum*-stimulated lymph node cells was significantly higher than that of pooled lymph nodes from normal mice. Treated cells showed no reduced response to an optimal PHA dose but a ten-fold increase in PHA concentration almost abolished the response of these cells. As with *C-parvum*-treated spleen cells, there was again evidence of cytotoxicity with the highest PHA dose.

d. Peripheral blood lymphocytes (Fig. 1D). Blood lymphocytes from *C. parvum*-treated mice showed markedly decreased PHA responsiveness compared with cells from normal animals.

Kinetics of PHA responsiveness of C. parvum-treated spleen cells. The routine culture procedure, pulsing with thymidine after 48 hr, might have missed any premature response by the *C. parvum*-treated cells. To exclude this possibility, treated and normal spleen cells were incubated with or without the optimal PHA dose

(0.001 ml/ml MEM) and ^{14}C-T added at 6, 18, 30 and 54 hr. Cultures were terminated 18 hr after addition of the isotope (Fig. 2). PHA stimulated a detectable increase of the DNA synthesis by normal cells between 36–48 hr, but no premature response on the part of the *C. parvum*-treated cells was observed.

PHA responsiveness of increasing numbers of C. parvum-treated spleen cells. The optimal response of normal spleen cells to a particular dose of PHA is dependent on cell density (13). A remaining possibility therefore was that, during the splenomegaly induced by *C. parvum* treatment, there had been a division of non-PHA responsive cells (B cells and macrophages) which had effectively diluted the PHA responsive cells. If this were the case, then one might expect to detect such a residual population by increasing the number of cells in the test system. Increasing numbers of cells were stimulated with a PHA dose which was optimal for 10^6 cells (Fig. 3). While the normal cells produced the expected response curve, no residual PHA-reactive cells among *C. parvum*-treated spleen cells were revealed.

PHA responsiveness of spleen cells after treatment with C. diphtheriae. Corynebacterium parvum is not only an adjuvant but also a potent immunogen. The fol-

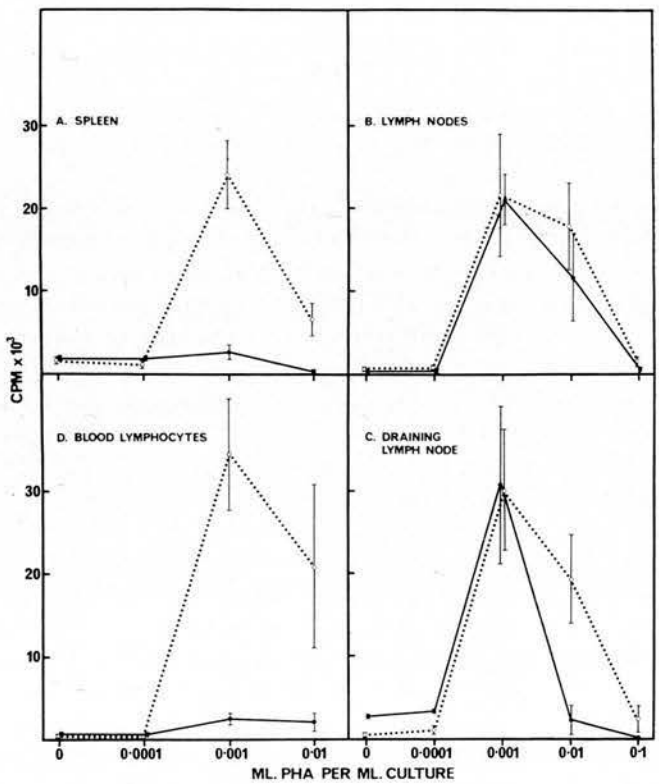

FIG. 1. PHA responsiveness of lymphocytes from mice injected with *C. parvum*. Cells from normal mice (○ • • • • ○), cells from *C. parvum*-treated mice (●——●). Spleen cells, pooled lymph node cells and peripheral blood lymphocytes (A, B, D) were assayed 7 days after iv injection of *C. parvum*. Draining lymph node (popliteal) cells (C) were assayed 7 days after footpad injection of *C. parvum*. Vertical bars represent ± standard error ($n = > 5$).

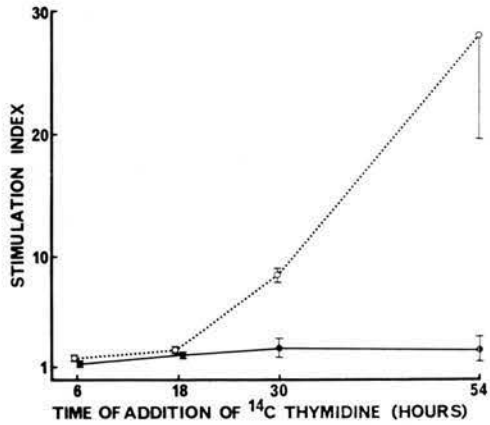

FIG. 2. Kinetics of the PHA responsiveness of spleen cells from normal mice (○ • • • ○) and those which had received *C. parvum* iv 7 days previously (●——●). Vertical bars represent ± standard error ($n = 3$).

lowing experiments were designed to determine whether the refractory state of *C. parvum*-treated cells was merely part of the general response to a recent dose of bacterial antigen or whether it was peculiar to *C. parvum* with its known immunostimulatory properties.

Corynebacterium parvum and *C. diphtheriae* are equally immunogenic in mice on

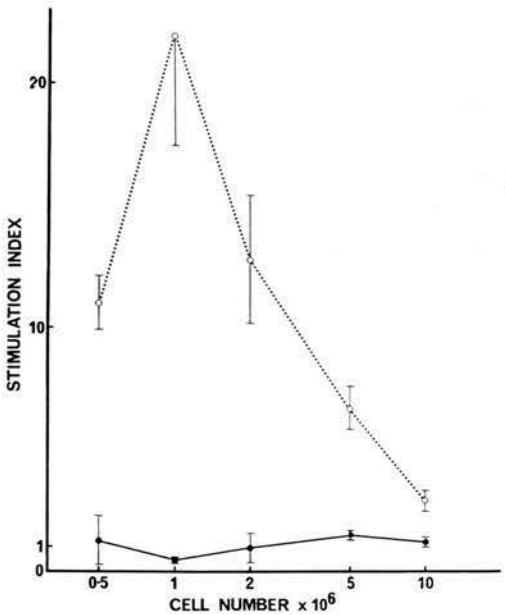

FIG. 3. The response of varying numbers of spleen cells from normal mice (○ • • • ○) and those which had received *C. parvum* iv 7 days previously (●——●) to a dose of PHA optimal for 1×10^6 spleen cells. Vertical bars represent ± standard error ($n = 3$).

the basis of the primary bacterial agglutinin response to a comparable dose of each (Scott, unpublished results). *Corynebacterium diphtheriae,* however, has no immunostimulatory properties as assessed by splenomegaly, adjuvanticity, increased carbon clearance (12) and antitumour activity (Scott, unpublished results). The PHA response of spleen cells from mice injected intravenously 7 days previously with an equivalent amount (dry weight) of *C. diphtheriae* was tested (Fig. 4). No significant difference in PHA responsiveness was found between *C. diphtheriae*-treated and normal spleen cells. The PHA phenomenon observed after *C. parvum* treatment would therefore seem to be associated with the immunostimulatory property of this organism.

Mixed lymphocyte reactivity of C. parvum-treated spleen cells. In view of the apparent inability of *C. parvum*-treated spleen cells to respond to PHA, another function of T-cells was next examined—their ability to respond to histocompatibility antigens *in vitro* in a mixed lymphocyte reaction (MLR).

One way MLRs were set up with normal CBA-*p* spleen cells or *C. parvum*-treated spleen cells reacting against irradiated DBA/2 normal spleen cells. Control cultures contained irradiated normal CBA-*p* spleen cells. Stimulation, as judged by increased DNA synthesis of mixed cultures compared with control cultures, occurred with both normal and *C. parvum*-treated cells, but stimulation was consistently less with *C. parvum*-treated cells (Table 1).

GVH reactivity of C. parvum-treated spleen cells. The GVH reactivity is another known function of T-cells and, in the light of the PHA and MLR results, *C. parvum*-treated cells were tested for their ability to cause a GVH reaction.

Spleen cells from normal C57BL mice and those which had received *C. parvum*

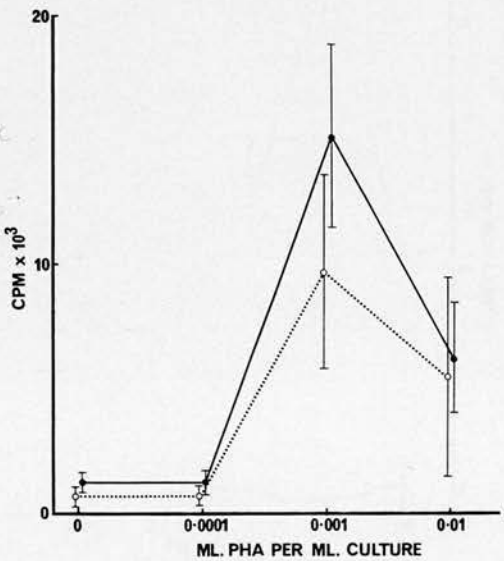

FIG. 4. PHA responsiveness of spleen cells from normal mice (○ • • • ○) and those which had received *C. diphtheriae* iv 7 days previously (●——●). Vertical bars represent ± standard error ($n = 3$).

TABLE 1

REACTIVITY OF *C. parvum*-TREATED SPLEEN CELLS IN A ONE WAY MIXED LYMPHOCYTE REACTION

Normal spleen cells	6.8[a]	2.3	6.2	9.8
C. parvum-treated spleen cells	2.6	1.3	2.6	3.2
% reduction[b]	38.2	56.5	41.9	32.7

[a] Stimulation index: cpm CBA-*p* + irradiated DBA/2/cpm CBA-*p* + irradiated CBA-*p*.

[b] Stimulation index of *C. parvum*-treated cells expressed as percentage of stimulation index normal cells.

7 days previously were injected either intraperitoneally or intravenously into (CBA-T6T6 × C57BL)F_1 mice. To control for any splenomegaly that may result from a carry over of persisting *C. parvum* with the cells, syngeneic cell transfers from both normal and *C. parvum*-treated F_1 mice were included (Table 2). Indeed, a significant degree of splenomegaly (iv $P = < 0.05$, ip $P = < 0.001$) did result from such transfers indicating persistence of intracellular *C. parvum* after 7 days. *Corynebacterium parvum*-treated cells did, however, cause GVH splenomegaly in the parent→ F_1 transfers, although this was considerably less than that caused by normal cells.

The response of C. parvum-treated spleen cells to pokeweed mitogen (PWM). The phytomitogen PWM has been demonstrated to stimulate both T- and B-lymphocytes (13, 14). Since the T-cells of *C. parvum*-treated spleens were shown to be unresponsive, the PWM response of such cells should give some indication of the status of the B-cell population with respect to their ability to be stimulated.

The PWM responsiveness of *C. parvum*-treated spleen cells was markedly depressed compared to spleen cells from normal mice (Fig. 5). However, since PWM stimulates both B- and T-cells, and the T-cells are already deemed to be unresponsive, it may be that the decreased PWM response after *C. parvum* merely

TABLE 2

G-V-H REACTIVITY OF *C. parvum*-TREATED SPLEEN CELLS IN (CBA–T6T6 × C57BL)F_1 ADULT MICE

Dose of spleen cells injected	Donor spleen cells			
	Syngeneic (CBA–T6T6 × C57BL)F_1		Parental C57BL	
	Normal	*C. parvum*-treated	Normal	*C. parvum*-treated
10^8 ip	39.4 ± 2.1[a]	45.7 ± 1.9	96.4 ± 5.9 (2.4)[b]	61.8 ± 2.2 (1.4)
5×10^7 iv	42.1 ± 0.9	52.1 ± 0.8	109.0 ± 4.9 (2.6)	89.9 ± 4.9 (1.7)

[a] Relative spleen weight (mg/10 g body wt) ± SE of recipient F_1 mice 9 days after spleen cell injection (16 mice per group).

[b] Relative spleen index–relative spleen weight with parental cells/relative spleen weight with F_1 cells.

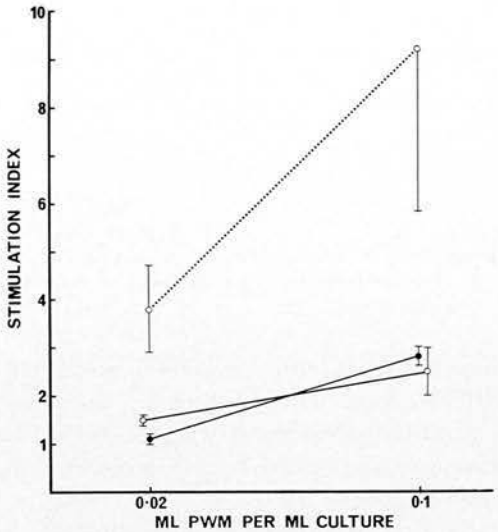

FIG. 5. PWM responsiveness of spleen cells from normal mice (○ • • • • ○), T-cell-deprived mice (○——○) and mice which had received *C. parvum* iv 7 days previously (●——●). Vertical bars represent ± standard error ($n = 6$).

reflects the residual B-cell response. In order to resolve this the PWM response of spleen cells of T-cell-deprived mice was assayed and found to be not significantly different from *C. parvum*-treated cells (Fig. 5).

DISCUSSION

Spleen cells and peripheral blood lymphocytes from mice pretreated with intravenous bacterial adjuvant, *C. parvum,* have been demonstrated to be markedly unresponsive or refractory to stimulation by PHA. It seems likely that the unaltered PHA response of lymph node cells after intravenous *C. parvum* is because the majority of particulate matter injected via this route is rapidly removed from the bloodstream and localised in the macrophages of the liver and spleen. Indeed, although intravenous *C. parvum* caused marked splenomegaly and hepatomegaly, no significant increase in the weight of a representative (inguinal) lymph node was detectable (Scott, unpublished results). If the PHA effect is a local one dependent on the presence of *C. parvum* then the "intermediate" results obtained with the popliteal lymph node cells after footpad injection may be reconciled with a relatively small amount of *C. parvum* being present in the node after footpad injection. A marked increase in lymph node weight was always evident after treatment but an element of this may be attributed to trauma at the injection site.

A prerequisite for the understanding of the mode of action of immunological adjuvants would seem to be the determination of which of the cells involved in the immune response are affected by them. The ability to respond to PHA is a property of T-lymphocytes (7) and the present findings thus indicate this to be one type of lymphocyte at least which is affected by the adjuvant *C. parvum*. It seems paradoxical, however, that treatment which is known to enhance T-cell-mediated

processes should result in inhibition of PHA responsiveness. At this stage it can only be conjectured that *C. parvum* has in some way, nonspecifically stimulated the T-cells, rendering them incapable of responding to a further mitogen.

That *C. parvum* affects T-cells is interesting in the light of the recent finding of the T-cell dependence of potentiation of antibody formation by a variety of adjuvants, including the bacterial vaccine *Bordetella pertussis* (15). Further evidence for T-cell involvement in adjuvant expression comes from the histological studies of Taub *et al.* (16) who demonstrated hypercellularity in the thymus-dependent area of draining lymph nodes after footpad injection of both antigenic and non-antigenic adjuvants. Synthetic polynucleotide adjuvants have also been reported to act on a T-cell population (17) although there is contrary evidence that the B-cell is the target for such adjuvants (18).

The finding that mixed lymphocyte and GVH reactivity, both T-cell functions, are reduced after *C. parvum* treatment is in keeping with the lack of PHA responsiveness. It is possible that the actual reduction in GVH reactivity may be less than indicated by the data in Table 2, since some persisting *C. parvum* has been shown to be transferred with the cells. Howard *et al.* (19), using the same GVH system, demonstrated that pretreatment of F_1 mice with *C. parvum* abrogated GVH in terms of reduced splenomegaly. What is not known in the present system is whether the amount of *C. parvum* transferred with the spleen cells is sufficient to be effective in this manner. That some MLR and GVH reactivity was still detectable despite the generally complete lack of PHA responsiveness may be reconciled with the fact that rat lymphocytes which participate in MLR and GVH may be separated from PHA-reactive cells on density gradients (20). The MLR of mouse spleen cells has also been demonstrated to be reduced after treatment with anti-θ serum and complement, without affecting PHA responsiveness (21).

The poor PWM response of T-cell-deprived mice shows that PWM in this system was not very effective at stimulating B-cells. The similarity between the response of cells from *C. parvum*-treated mice and T-cell-deprived animals suggests that the depressed PWM response after *C. parvum* is not due to an effect of *C. parvum* on the B-cells. However, the recent demonstration by Howard (personal communication) that *C. parvum* pretreatment has an adjuvant effect with the thymus-independent antigen pneumococcal polysaccharide type 3 (SIII) might suggest at least a degree of B-cell involvement.

To date there appears to have been a fairly good correlation between the PHA and MLR responsiveness of lymphocytes and over-all immune status, cell-mediated in particular (8–10, 22). The present finding of drastically reduced responsiveness of lymphocytes from animals with elevated cellular and humoral immune status urges caution concerning the validity of such tests as measures of immunocompetence under some circumstances.

The mechanism of the depression of PHA responsiveness after *C. parvum* has been the subject of further study and the results and their significance are set out in the following paper (23).

It has since been demonstrated that E. Coli endotoxin stimulates exclusively B cells (Gery *et al., J. Immunol.* **108**, 1088, 1972). Subsequent experiments have shown no difference in the endotoxin response of spleen cells from normal and

C. parvum-treated mice, providing further evidence for no effect of C. parvum on B. cells.

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Biological Effects of the Adjuvant *Corynebacterium Parvum*

II. Evidence for Macrophage–T-Cell Interaction

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The mechanism underlying the markedly reduced PHA responsiveness of spleen and peripheral blood lymphocytes from *Corynebacterium parvum*-treated mice is due to inhibition of the responsive T-lymphocytes by *C. parvum*-activated macrophages. Inhibition is a result of a qualitative rather than quantitative change in the macrophage population and GVH-activated macrophages behave similarly. *Corynebacterium parvum*-activated macrophages need to be viable and will inhibit normal lymphocytes. This inhibitory effect appears to be mediated through cell–cell contact.

INTRODUCTION

Spleen cells and blood lymphocytes from mice pretreated with the bacterial adjuvant *Corynebacterium parvum* have been found to be refractory or markedly unresponsive to stimulation by phytohaemagglutinin (PHA) *in vitro* (1). As other recognised T (thymus-derived) cell functions [mixed lymphocyte and graft-versus-host (GVH) reactivity] were similarly impaired, it was concluded that the T-lymphocyte is affected by this adjuvant. The present study concerns the nature of the PHA unresponsiveness and presents evidence for macrophage–T-cell interaction under the influence of *C. parvum*.

MATERIALS AND METHODS

Animals. Adult mice of the inbred strain CBA-*p* were used throughout.

Bacteria. Corynebacterium parvum suspension (Batch No. EZ174, 7 mg/ml) was provided by the Wellcome Research Laboratories, Beckenham, Kent, England. A standard dose of 0.2 ml was injected intravenously.

Cell suspensions and the response to PHA. The preparation of lymphoid cell suspensions and their responsiveness to PHA assayed by [^{14}C]-thymidine incorporation are described in detail in the preceding paper (1). Any deviations from the routine procedure are described in the text. Except where stated, PHA was used at the optimally stimulating dose of 0.001 ml/ml culture.

Column filtration of cell suspensions. Water-jacketed glass columns (26 cm × 1 cm) were filled with glass beads (3 mm diam). The volume when filled was 10–11

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ml. Spleen cells (10 ml) (10^7/ml) in Eagles Minimum Essential Medium (MEM) containing 10% foetal calf serum were applied to the column and allowed to adhere for 15 min at 37°C. The nonadherent cell population was eluted by addition of 10 ml of fresh serum containing medium at 37°C, centrifuged and resuspended at 10^6/ml ready for culture. Peripheral blood lymphocytes were fractionated similarly using fewer cells in the same final volume.

Magnetic separation of phagocytic cells. The technique described by McCullagh (2), with slight modification, was used. Spleen cells were incubated in centrifuge tubes with carbonyl iron powder for 1 hr at 37°C. The cells were resuspended and the tube drawn upward between the poles of a powerful magnet, the resulting supernatant being transferred to another centrifuge tube. This process was repeated six times. The cells remaining in the supernatant were centrifuged and resuspended in MEM containing serum at 10^6/ml.

Carbon clearance. The rate of clearance from the blood of intravenously injected colloidal carbon (0.1 ml/g body wt of 16 mg/ml suspension) was as described by Biozzi *et al.* (3). Groups of eight mice were used.

GVH mice. Adult (CBA-T6T6 × C57BL)F_1 mice were injected intravenously with 10^8 C57BL spleen cells. Spleen weights were markedly increased at 8 days, when spleens were removed and cell suspensions prepared.

EXPERIMENTAL

The effect of column filtration on normal and C. parvum*-treated spleen cells.* Spleen cells from normal mice and animals injected intravenously with 0.2 ml *C. parvum* suspension 7 days previously were compared for their ability to respond to PHA after removal of adherent cells by glass bead column fractionation (Fig. 1). Unfractionated *C. parvum*-treated (CP) spleen cells were totally unresponsive to PHA stimulation as described previously (1). The nonadherent cell populations of such spleens were, however, fully responsive to PHA. Although removal of adherent cells from normal spleens resulted in somewhat higher mean PHA responsiveness, this was not statistically significant at any of the doses tested.

Effect of removal of phagocytic cells on C. parvum*-treated spleen cells.* Glass adherence is a generally accepted procedure for the removal of phagocytic cells from various cell populations, although some nonphagocytic cell types adhere to glass after antigen stimulation *in vivo* (4). As *C. parvum* is a potent immunogen as well as an adjuvant (Scott, unpublished results) it seemed desirable to use a more discriminative technique for removal of phagocytic cells—magnetic separation.

The effect of removing phagocytic cells with carbonyl iron was similar to column fractionation (Fig. 2) and PHA responsiveness was again restored to a level comparable with normal spleen cells. Unlike column fractionated cells, the isotope incorporation into unstimulated iron-fractionated cells was consistently higher than into unstimulated whole cells.

The effect of column filtration on C. parvum*-treated blood lymphocytes.* Peripheral blood lymphocytes isolated from mice which had received *C. parvum* intravenously 7 days previously were fractionated and the nonadherent cell population tested for PHA responsiveness compared with unfractionated cells (Table 1). The PHA response of lymphocytes from CP mice, as compared with normal animals,

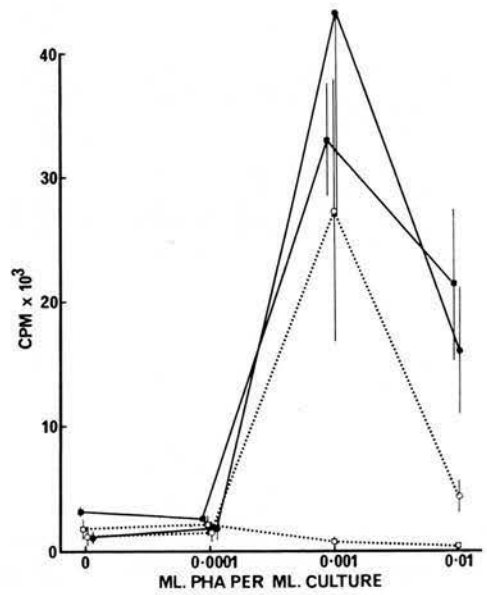

FIG. 1. The effect of the removal of glass-adherent cells by glass bead filtration on the PHA responsiveness of normal spleen cells and those from mice which had received *C. parvum* intravenously 7 days previously. Normal spleen cells ○ • • • • ○, filtered normal spleen cells ●——●, *C. parvum*-treated spleen cells □ • • • • □, filtered *C. parvum*-treated spleen cells ■——■. Vertical bars represent ± standard error ($n = 3$).

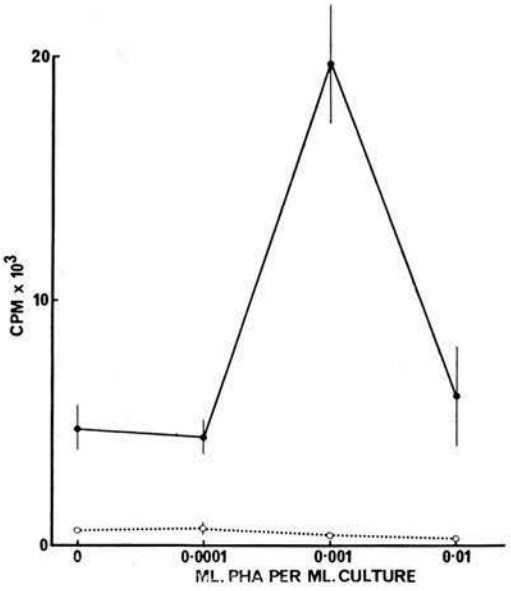

FIG. 2. The effect of the removal of phagocytic cells by magnetic separation on the PHA responsiveness of spleen cells from mice which had received *C. parvum* intravenously 7 days previously. Whole cells ○ • • • • ○, fractionated cells ●——●. Vertical bars represent ± standard error ($n = 3$).

TABLE 1

THE EFFECT OF GLASS BEAD FILTRATION OF BLOOD LYMPHOCYTES FROM *C. parvum*-TREATED MICE ON THEIR PHA RESPONSIVENESS

	Blood lymphocytes		
	Normal	*C. parvum*-treated	*C. parvum*-treated filtered
cpm: Unstimulated	388.0 ± 135.6 [a]	350.3 ± 64.3	546.9 ± 188.7
cpm: PHA-stimulated	29,879.1 ± 4942.5	3762.0 ± 225.7	12,855.2 ± 2695.0
Stimulation index [b]	104.1 ± 19.9	11.7 ± 2.7	25.5 ± 2.9

[a] Mean ± standard error ($n = 5$).
[b] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

was greatly reduced as anticipated. Removal of glass-adherent cells from the CP population consistently resulted in increased PHA responsiveness. However, in contrast with spleen cells, the degree of PHA responsiveness restored to blood lymphocytes was never complete, in that stimulation remained well below that of unfractionated normal blood lymphocytes.

The nature of macrophage-mediated inhibition of lymphocyte PHA responsiveness. The implication of the fractionation experiments is that potentially PHA-responsive lymphocytes are present in CP spleens and peripheral blood but are inhibited by the presence of phagocytic cells. *Corynebacterium parvum* has a stimulatory effect on macrophages evidenced both histologically and by increased carbon clearance (5). Is the inhibition phenomenon due merely to the presence of increased numbers of macrophages or does it result from some qualitative change in these cells? It has been reported that an excess of macrophages in some *in vitro* immunological systems exerts an inhibitory influence (6, 7, 8).

The proportion of glass-adherent cells in both normal and CP spleens was compared quantitatively by adding equal numbers (10^8) of spleen cells to columns and counting the numbers of nonadherent cells eluted under identical conditions. The yields from seven comparisons of normal and CP spleens were not significantly different, being $4.01 \pm 0.31 \times 10^7$ (± standard error) and $3.8 \pm 0.44 \times 10^7$, respectively. On this basis the inhibition phenomenon would appear to be attributable to a qualitative rather than a quantitative change in the spleen macrophage population.

Inhibition of PHA responsiveness of normal lymphocytes by CP spleen cells. As a first step toward analysing the inhibition phenomenon in greater detail, CP spleen cells were added to normal spleen or lymph node cells and the mixtures assayed for any reduction in PHA responsiveness. Complete inhibition resulted when normal and CP cells were mixed in the ratio 1:1 (Table 2).

If the implication of the column experiments that the inhibiting cells are glass-adherent is correct, then this population alone should be inhibitory.

It proved impossible to elute glass-adherent cells retained during column filtration with versene, since it caused aggregation during centrifugation. The cells were recovered in the following manner: 1-ml aliquots (2×10^6) normal or CP spleen

TABLE 2

INHIBITION OF PHA RESPONSIVENESS OF NORMAL SPLEEN CELLS BY ADDITION OF *C. parvum*-TREATED SPLEEN CELLS

	Normal cells	*C. parvum*-treated cells	Normal + *C. parvum*-treated cells
cpm: Unstimulated	1363 ± 379.7 [a]	807.6 ± 372.9	2515.1 ± 402.0
cpm: PHA-stimulated	15,152.6 ± 2812.2	867.6 ± 619.4	3605.6 ± 976.1
Stimulation index [b]	12.8 ± 2.8	0.8 ± 0.2	1.4 ± 0.2

[a] Mean ± standard error ($n = 3$).

[b] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

cells were incubated in culture tubes for 3 hr at 37°C. After gentle agitation, the overlying fluid and nonadhering cells were removed and replaced with 1 ml fresh medium with or without 10^6 normal spleen cells. These cultures were assayed for PHA reactivity in the standard manner. Preliminary experiments affirmed that the glass-adherent cells thus prepared from normal spleens were considerably less PHA-responsive than whole cells. Table 3 shows that the PHA response of normal spleen cells was almost completely inhibited by the presence of glass-adherent CP cells but not by those from normal spleens.

To test whether CP macrophages need to be alive to inhibit lymphocyte PHA responsiveness, suspensions of normal and CP spleen cells (2×10^6/ml) were killed by heating at 56°C for 30 min. (Cell death was confirmed by the absence of [^{3}H]-uridine incorporation during 3 hr after treatment.) 0.5 ml aliquots of killed cells or of cell-free supernatants of heat-killed cells prepared by centrifugation and Millipore filtration were added to 0.5 ml (10^6/ml) normal spleen cells. All cultures were assayed routinely for PHA responsiveness (Table 4).

There was no difference in the PHA response of normal spleen cells incubated with supernatant from either heat-killed normal or CP spleen cells. Although PHA stimulation of normal spleen cells was less when incubated with heat-killed CP cells than with heat-killed normal cells, the inhibition was minimal compared with the almost total inhibition with live CP cells (Table 2).

The effect of supernatants from unstimulated and PHA-stimulated CP spleen cells on the PHA response of normal lymphocytes. The absence of inhibition with supernatants from heat-killed CP cells does not exclude the possibility of soluble inhibitors that might be released from metabolising cells. A soluble human lymphocyte-derived factor which inhibits DNA synthesis after PHA stimulation has been described (9). Normal spleen cells were tested for their PHA responsiveness when grown in medium from either normal or CP spleen cells cultured with or without PHA for 3 days. These media were prepared by centrifuging the 3-day cultures at 2×10^3 rpm for 10 min at 0°C and Millipore filtering the supernatants; 0.9 ml aliquots of the supernatants were dispersed into fresh culture tubes and 0.1 ml (10^6) normal spleen cells added. These cultures were then assayed for PHA responsiveness (Table 5). There was no difference in response between cells incubated in supernatants from normal or CP spleen cell cultures. The stimulation indices for cells grown in media from PHA-stimulated cultures were less because of

TABLE 3

INHIBITION OF PHA RESPONSIVENESS OF NORMAL SPLEEN CELLS BY THE GLASS ADHERENT FRACTION OF *C. parvum*-TREATED SPLEEN CELLS

	Glass adherent normal spleen cells		Glass adherent *C. parvum* spleen cells	
	Alone	+ Whole normal spleen cells	Alone	+ Whole normal spleen cells
cpm: Unstimulated	586.0 ± 37.0 [a]	3252.8 ± 390.3	1424.0 ± 267.5	3929.5 ± 279.0
cpm: PHA-stimulated	2642.0 ± 936.5	31,409.5 ± 285.0	910 8 ± 482.3	7253.8 ± 971.3
Stimulation index [b]	4.6 ± 1.9	9.7 ± 0.2	0.6 ± 0.2	1.9 ± 0.2

[a] Mean ± standard error ($n = 3$).
[b] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

TABLE 4

THE EFFECT OF HEAT-KILLED *C. parvum*-TREATED SPLEEN CELLS ON THE PHA RESPONSIVENESS OF NORMAL SPLEEN CELLS

	Normal spleen cells cultured with			
	Supernatant from killed normal cells	Supernatant from killed *C. parvum*-treated cells	Killed normal cells	Killed *C. parvum*-treated cells
cpm: Unstimulated	220.0 ± 61.5 [a]	198.0 ± 51.0	270.5 ± 52.5	208.8 ± 2.3
cpm: PHA-stimulated	12,601.5 ± 758.5	10,449.8 ± 879.3	11,446.5 ± 2519.5	5256.8 ± 2661.8
Stimulation index [b]	61.1 ± 13.6	57.8 ± 19.4	45.9 ± 18.3	25.3 ± 13.0

[a] Mean ± standard error ($n = 3$).
[b] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

TABLE 5

PHA RESPONSIVENESS OF NORMAL SPLEEN CELLS GROWN IN MEDIUM FROM 3-DAY CULTURES OF BOTH UNSTIMULATED AND PHA-STIMULATED *C. parvum*-TREATED SPLEEN CELLS

	Normal spleen cells grown in medium from			
	Normal spleen cells	*C. parvum*-treated spleen cells	PHA-stimulated normal spleen cells	PHA-stimulated *C. parvum*-treated spleen cells
cpm: Unstimulated	269.3 ± 41.8[a]	183.5 ± 41.5	1227.0 ± 606.5	661.5 ± 312.0
cpm: PHA-stimulated	3965.5 ± 1414.0	3434.3 ± 1805.8	4896.3 ± 1233.3	3910.5 ± 1649.5
Stimulation index[b]	14.3 ± 3.1	17.4 ± 5.9	4.6 ± 1.3	6.1 ± 0.4

[a] Mean ± standard error ($n = 3$).

[b] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

the residual activity of the PHA. However, cells incubated in supernatants from PHA-stimulated normal and PHA-stimulated CP cells responded equally well to freshly added PHA.

These results failed to demonstrate an inhibitory substance produced by CP spleen cells with or without the presence of PHA. They also exclude the possibility that failure to respond was merely due to depletion of essential nutrients. It is clearly impossible to exclude the release of an inhibitory substance which acts locally, but which becomes too diluted in the medium to be detectable.

Relationship between the inhibition phenomenon and carbon clearance. The inhibition of PHA responsiveness after injection of adjuvant *C. parvum* is attributable to a change in the macrophage population. Modification of macrophages after *C. parvum* is also evidenced by an over-all increase in phagocytic capacity (5). To see whether these two criteria for macrophage activity might be related, the rate of clearance of colloidal carbon and the PHA responsiveness of spleen cells were compared in parallel at varying times after *C. parvum* (Fig. 3).

Maximal increase in carbon clearance (threefold) was present 4 days after *C. parvum*. This fell by day 7, but was still maintained above background at day 21. PHA responsiveness was significantly reduced at day 4, but was not maximally depressed until day 7. Although it rose slightly by day 14, it remained markedly depressed at day 21. The different kinetics would seem to indicate a lack of identity between these parameters.

PHA responsiveness of GVH spleen cells. Similar intense reticuloendothelial stimulation to that induced by *C. parvum* also occurs during a GVH reaction (10). Certain striking similarities, considered in the Discussion, suggested that the GVH situation might provide a source of similarly activated macrophages. Eight-day GVH spleens were therefore examined for PHA responsiveness (Fig. 4) and found to be completely unresponsive. The controls shown in the graph are spleen cells from normal F_1 hybrids but mixtures of equal numbers of parental and F_1 cells were equally responsive. Removal of glass-adherent cells from GVH spleens resulted in the recovery of significant PHA responsiveness (Fig. 4), although this was minimal in comparison with the response of normal and filtered CP cells. However, as GVH disease is known to destroy lymphocytes preferentially (11),

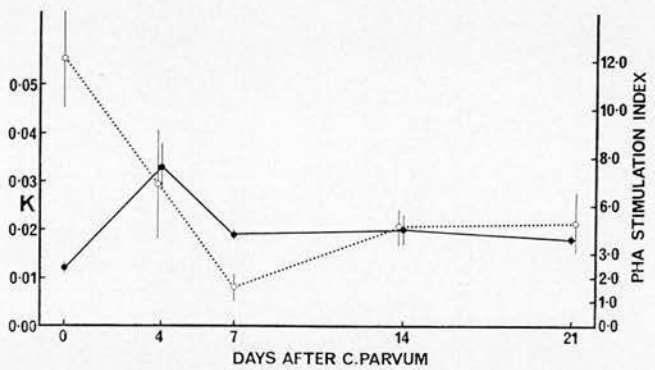

FIG. 3. Comparison of kinetics of increase in carbon clearance activity ●——● and depression of PHA responsiveness ○ • • • • ○ after intravenous *C. parvum*.

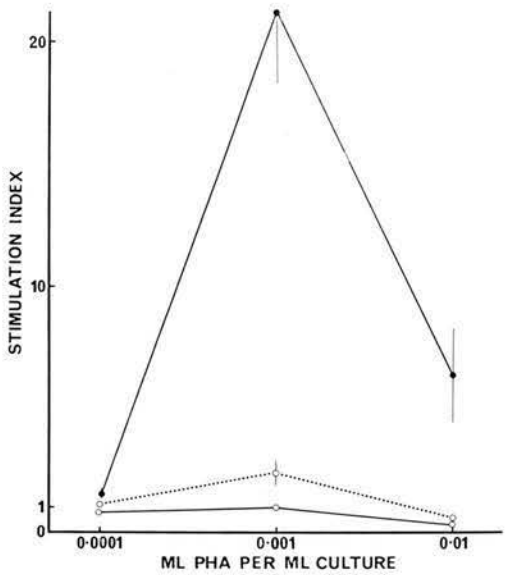

FIG. 4. The effect of removal of glass-adherent cells by glass bead filtration on the PHA responsiveness of spleen cells from mice undergoing GVH disease (10^8 C57BL spleen cells iv → (CBA-T6T6 × C57BL)F_1, spleens removed 8 days later). GVH cells ●——● filtered GVH cells ○••••○, normal F_1 hybrid cells ○——○. Vertical bars represent ± standard error ($n = 3$).

GVH spleens would be expected to be deficient in PHA-responsive cells. If, however, the GVH situation is analogous to *C. parvum* treatment, then GVH spleen cells ought to be capable of inhibiting the PHA responsiveness of normal lymphocytes. This prediction was verified (Table 6).

DISCUSSION

The present findings indicate that the inhibitory effect of *C. parvum* on the PHA responsiveness of T-lymphocytes is not a direct one but mediated by macrophages which have themselves been modified by the adjuvant.

TABLE 6

INHIBITION OF PHA RESPONSIVENESS OF NORMAL (CBA–T6T6 × C57BL)F_1 SPLEEN CELLS BY ADDITION OF GVH[a] SPLEEN CELLS

	Normal F_1 cells	GVH cells[a]	Normal F_1 + GVH cells
cpm: Unstimulated	392.7 ± 50.2[b]	366.7 ± 22.1	1197.7 ± 128.1
cpm: PHA-stimulated	7180.5 ± 747.9	471.7 ± 72.2	3803.5 ± 116.0
Stimulation index[c]	18.3 ± 1.9	1.3 ± 0.2	3.2 ± 0.1

[a] 10^8 C57BL spleen cells were injected intravenously into adult (CBA–T6T6 × C57BL)F_1 mice. Spleens were removed 8 days later.

[b] Mean ± standard error ($n = 3$).

[c] Ratio of cpm PHA-stimulated cells/cpm unstimulated cells.

There is evidence from the mixed lymphocyte reaction (6), *in vitro* graft reaction (7) and *in vitro* response to sheep cells (8), that although glass-adherent cells are necessary for these reactions, they are inhibitory when present in excess. Although macrophage proliferation occurs after injection of *C. parvum,* the results showed no disproportionate increase in the number of glass-adherent cells after *C. parvum* treatment.

The adjuvant activity of various substances correlates with their ability to labilise lysosomal membranes in macrophages (12) and some adjuvants exert a cytotoxic effect on macrophages (13). The adjuvant-stimulated macrophage is therefore likely to be in a delicate condition and more susceptible to any cytotoxic action of PHA than its normal counterpart. That the inhibition of the PHA response of lymphocytes is due to adverse culture conditions created by the breakdown products of dying macrophages is unlikely since heat-killed CP cells, their supernatants or supernatants from live CP cells were not inhibitory. For these reasons it is assumed that cytotoxic action of PHA on macrophages is not the inhibiting factor.

What then is the nature of the inhibition? The failure to inhibit with supernatants from CP spleen cells shows lack of identity between the present inhibiton and the soluble lymphocyte-derived factor described to inhibit PHA-stimulated DNA synthesis (9). The same experiment would also exclude any soluble inhibitory factors produced by the *C. parvum*-stimulated macrophages, thereby implying that inhibition must be due to cell–cell contact. The inefficacy of heat-killed cells indicates that the activated macrophages must be viable.

It is hardly surprising that any effect of *C. parvum* on lymphocytes is mediated by macrophages since the immediate fate of injected bacteria is to be phagocytosed and contained. The only direct contact between lymphocytes and *C. parvum* would presumably be via receptor sites for *C. parvum* on the lymphocyte surface, i.e., for *C. parvum* as an antigen. The adjuvant effect of a variety of substances appears to act selectively via the macrophage. In experiments with beryllium salt, *Bordetella pertussis* (14) and *Escherichia coli* lipopolysaccharide (15), treatment of lyphocytes with these adjuvants before using them to reconstitute irradiated recipients, gave no detectable adjuvant effect, whereas adjuvant processed by macrophages was effective. No over-all change in antigen handling by the stimulated macrophages was detectable and an effect other than that of antigen processing of presentation seemed likely (14, 15). Both groups of workers suggested that the most likely interaction between adjuvant-activated macrophages and immunocompetent lymphocytes was for the macrophage to stimulate proliferation of the lymphocyte population. Efficient antigen presentation would be insured by having the lymphocytes dividing and differentiating at the time of antigen presentation. It is difficult at this stage to reconcile such a stimulus with the present finding of inhibited PHA responsiveness unless perhaps macrophage stimulation of the T-lymphocyte in some way blocks stimulation with PHA. Even so, it is still unclear why no macrophage-stimulated lymphocyte proliferation is detectable *in vitro*. Askonas and Jaroskova (16) point out that such a stimulus could be effected by release of soluble stimulating factors or by membrane–membrane interaction. If, in fact, PHA inhibition after *C. parvum* treatment does represent lymphocyte stimulation by macrophages, the latter suggestion is correct.

There are many striking similarities between mice undergoing *C. parvum* stimulation and the proliferative stage of GVH disease. They show marked splenomegaly, increased carbon clearance (5, 10), raised sensitivity to endotoxin atttributed to increased sensitivity of the macrophages (17) and increased protection against bacterial infection due to macrophage activation (18, 19). That GVH spleen cells behave similarly to CP spleen cells with regard to PHA provides further evidence for the role of the macrophage in PHA inhibition and underlines the similarities between the two states.

The diverse nature of substances expressing adjuvant activity would seem to exclude any unified hypothesis of adjuvant action. There is, however, accumulating evidence that the cell type primarily affected by a variety of adjuvants is the macrophage and this has now been demonstrated to be true for *C. parvum*. Although, at this stage, the *in vivo* correlate of *C. parvum*-activated macrophage inhibition of lymphocyte PHA responsiveness must remain conjectural, it may constitute a cell–cell interaction peculiar to stimulated conditions. Should this be the case, it would seem to be a useful assay for activated macrophages.

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Biological Effects of *Corynebacterium parvum*

IV. Adjuvant and Inhibitory Activities on B Lymphocytes

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The PFC response to the thymus-independent antigen SIII (type 3 pneumococcal polysaccharide) was amplified in mice injected 4 days previously with killed *Corynebacterium parvum*. This adjuvant activity was demonstrable with high (2–50 μg) but not low (0.1–0.5 μg) doses of SIII. Induction of tolerance was unaffected. Depression of the response resulted from simultaneous injection of SIII with either *C. parvum* or *Bordetella pertussis*, while prior treatment with the latter was without effect. Responsiveness to SIII was transiently but potently suppressed in spleen cells transferred into lethally irradiated, *C. parvum* pretreated mice.

Although *C. parvum* is an effective B cell adjuvant, other data imply that it acts indirectly on these lymphocytes. It is argued that both adjuvant and suppressive activities of *C. parvum* on the B cell response to SIII are most probably mediated by activated macrophages.

Investigations on the mode of action of adjuvants have recently centered around which type of lymphocyte, the thymus-derived (T) cell or the bursa-equivalent (B) cell, is the site of stimulation. An obligatory role for T cells in the adjuvant activity of *Bordetella pertussis* has been argued on the basis of studies with thymus-deprived mice (1, 2). This view has been qualified by the large-scale investigation of Dresser (3) who found that the stimulating effect of *B. pertussis* was directly on B cells when large doses of antigen were used, whereas amplification of responses was mediated via T cells with smaller doses. Conflicting reports which have appeared concerning the class of lymphocyte affected by polynucleotide adjuvants (4–6) may have a similar basis.

Corynebacterium parvum is a potent stimulant of the reticuloendothelial system (7) and also possesses adjuvant (8) and antitumor activities *in vivo* (9). Stimulation with *C. parvum* is accompanied by a range of depressed T cell activities (10) attributable to the direct influence of activated macrophages (11). The present communication describes and analyzes the effects of *C. parvum* on the B cell response to the strictly thymus-independent antigen SIII (type 3 pneumococcal polysaccharide).

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MATERIALS AND METHODS

Mice. Adults of the CBA-T_6T_6 inbred strain were used throughout. In any experiment, mice were of the same sex and did not vary in weight by more than 5 g.

Antigens. The preparation and properties of the following antigens used have been published previously: SIII (12); ^{14}C-labeled SIII (13); Levan (14). Sheep erythrocytes (SE) were supplied by these laboratories.

Corynebacterium parvum. A standard suspension of killed *C. parvum* (strain CN6134) with a concentration of 7 mg/ml (batch EZ 174) was prepared in these laboratories.

Bordetella pertussis. A standard suspension (VC 3148) of killed bacteria with a concentration of 8×10^{10} per ml was supplied by these laboratories.

Spleen cell suspensions were prepared by a conventional procedure (15).

Plaque-forming cell assays. Direct PFC counts on spleen cells were used throughout as a measure of the response to SIII, which is solely IgM in the CBA mouse. The technique using SE sensitized with type 3 pneumococcal culture filtrate (16) has been described in detail elsewhere (17). Direct PFC assays using levan and SE were according to the techniques described by Miranda (14) and Dresser and Wortis (18), respectively.

Thymectomy. Adult mice were surgically thymectomized or sham-operated (19).

Irradiation. Mice received a standard dose of 900 R whole-body irradiation from a ^{60}Co source with a dose rate of approximately 80 R/min. and a focal distance of 18 cm.

Clearance of ^{14}C-SIII from the circulation. Estimations of the levels of ^{14}C-SIII in the serum were made using a Beckman liquid scintillation counter. The techniques and calculations involved have been described previously (13).

In vitro *stimulation of spleen cells with mitogens.* The effect on T and B lymphocytes was studied with phytohemagglutinin (PHA) and *E. coli* lipopolysaccharide (LPS), respectively (20). The preparation of spleen cell suspensions, their culture and stimulation with PHA have been described previously (10). LPS from *E. coli*

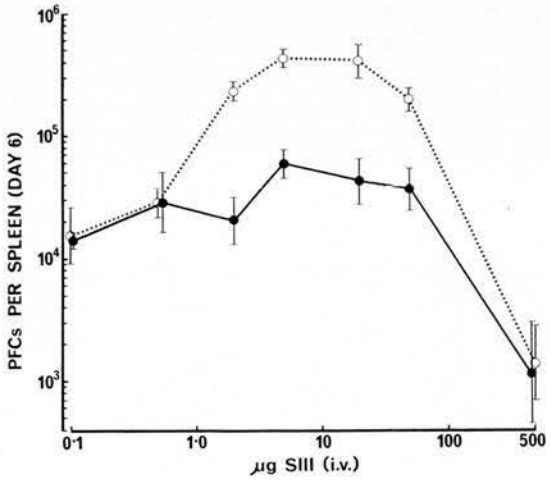

FIG. 1. Effect of *C. parvum* pretreatment on the PFC response in the spleen 6 days after various doses of SIII. ●——● Normal mice. ○····○ Mice injected with 0.7 mg *C. parvum* 4 days before SIII. Standard errors indicated ($n = 5$).

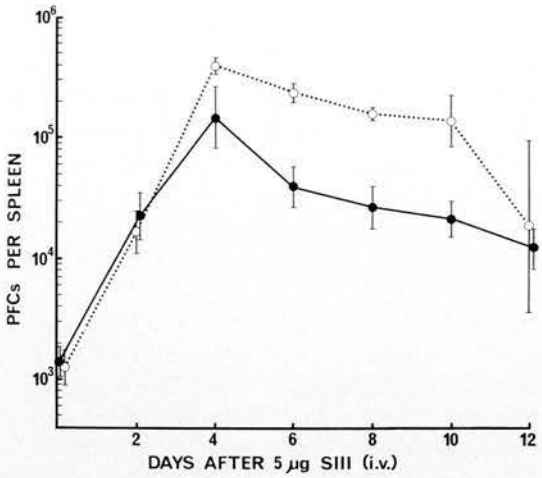

FIG. 2. Effect of *C. parvum* pretreatment on the course of the PFC response in the spleen to 5 μg SIII. ●——● Normal mice. ○····○ Mice injected with 0.7 mg *C. parvum* 4 days before SIII. Standard errors indicated ($n = 5$).

extracted by the hot phenol–water method was kindly provided by Dr. L. Nagy of these laboratories. Prior to use 1 mg/ml was dissolved in culture medium and 0.1-ml aliquots of various dilutions added to the cultures. As with PHA, ^{14}C thymidine was added 48 hr later and the cultures terminated after a further 24 hr for assay of isotope incorporation (10). The results are expressed as a stimulation index: the ratio of cpm in experimental culture/cpm in control culture.

RESULTS

The Adjuvant Effect of C. parvum *on the B Cell Response to SIII*

Groups of five mice which had been pretreated with 0.1 ml *C. parvum* iv 4 days previously were challenged with various doses of SIII iv and the PFC responses in the spleen compared after 6 days with those in normal mice (Fig. 1). No adjuvant effect of *C. parvum* was detectable with small immunizing doses of SIII (0.1 and 0.5 μg), whereas a one-order increase in PFC level was detectable using larger doses (2, 5, 20, and 50 μg). Induction of tolerance by 500 μg SIII was not impaired by *C. parvum* treatment. The time course of the augmented response to 5 μg SIII is shown in Fig. 2. The increase in PFC on Day 2 was similar in both *C. parvum*-treated and normal mice, but showed divergence thereafter, the former group reaching a higher peak and substaining a greater response through Days 4–10. Although *C. parvum* produced an average 3-fold increase in the spleen cell count, this experiment implies that its adjuvant effect involves an increase neither in the number of SIII-reactive B cells nor the initial rate of proliferation of PFCs.

It is obligatory to inject *C. parvum* several days before antigen to develop an adjuvant effect. Simultaneous administration with SIII was found to reduce significantly the PFC response (Fig. 3). The effect of injecting 2×10^9 *B. pertussis* (iv) with 5 μg SIII was also studied, as this adjuvant is conventionally given together with antigen. Again, some depression of the PFC response was found (Fig. 3), although the dose of *B. pertussis* used effectively augmented the response to 5×10^8 sheep erythrocytes. This inhibitory activity was even more potent in another experi-

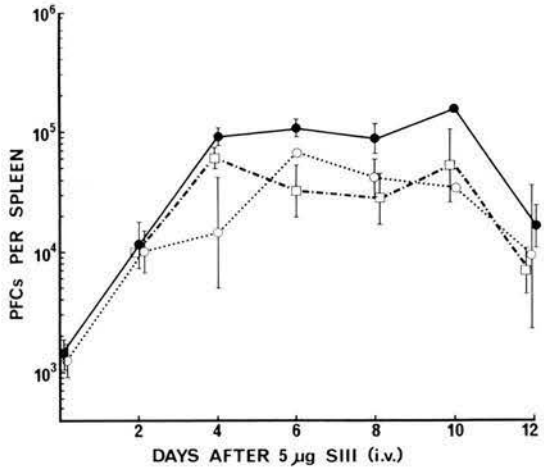

FIG. 3. Effect of simultaneous administration of *C. parvum* or *B. pertussis* with 5 μg SIII on the PFC response in the spleen. ●——● Normal mice. ○····○ Mice given 0.7 mg *C. parvum*. □-·-·-□ Mice given 2×10^9 *B. pertussis*. Standard errors indicated ($n = 5$).

ment in which the effects on the response to 5 μg SIII of prior or simultaneous treatment with *B. pertussis* were compared. The following PFC counts ($\log_{10} \pm$ SE with antilog) were obtained on Day 6: no adjuvant 5.030 ± 0.124 (107,150), *B. pertussis* 4 days before SIII 5.107 ± 0.094 (127,940), *B. pertussis* with SIII 3.870 ± 0.114 (7,415). Thus, no adjuvant effect was obtained by pretreatment, while simultaneous injection with SIII was highly inhibitory to the immune response to this antigen.

The results in Fig. 2 suggest that *C. parvum* treatment does not induce an intrinsic change in the B lymphocyte population, but rather that the augmented response to SIII was attributable to their stimulation by some extrinsic influence. This view is

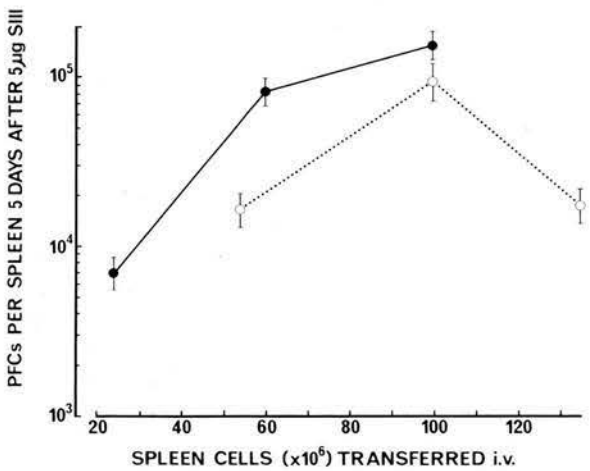

FIG. 4. Failure of *C. parvum*-induced adjuvant effect on B cells to transfer adoptively. PFC response to 5 μg SIII in 900-R irradiated mice repopulated with various doses of syngeneic spleen cells from normal donors (●——●) or mice injected with 0.7 mg *C. parvum* 4 days previously (○····○). (Standard errors indicated, $n = 5$).

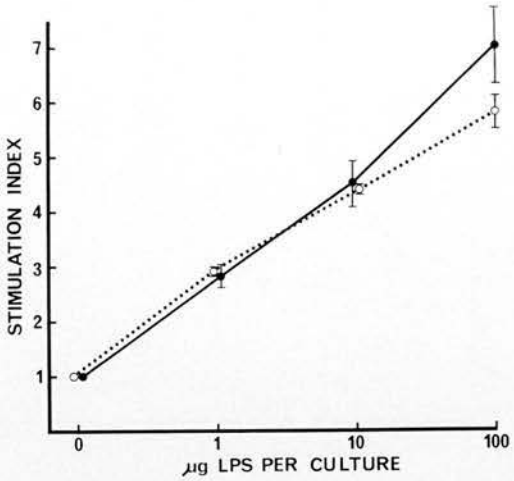

FIG. 5. Responsiveness to LPS of spleen cells from normal mice (●——●) and mice injected with *C. parvum* 4 days previously (○···○). (Standard errors indicated, $n = 3$).

supported first by the result of cell-transfer experiments. Various doses of spleen cells from *C. parvum*-treated and normal mice were transfered to groups of 900 R irradiated mice which were then challenged with 5 μg SIII (Fig. 4). Up to 50% more cells from *C. parvum*-treated donors were required to confer equivalent responsiveness, commensurate with the increased cellularity of the spleens. (The highest cell dose transferred (130×10^6) showed a reduction in efficacy, the basis for which has not yet been sought.) Secondly, spleen cells from normal and *C. parvum*-treated mice responded similarly *in vitro* to stimulation by the B cell mitogen *E. coli* LPS (Fig. 5).

Various experiments were carried out to see whether the stimulatory effect of *C. parvum* on the B cell response to SIII might be mediated by either T cells or macrophages as (a) the SIII response is augmented by T cell activity in GVH reaction (21) and (b) macrophages suppress some T cell activities in *C. parvum*-stimulated animals (11).

Results from the use of conventional thymus-deprived mice were inconclusive. Adult thymectomized animals, lethally irradiated, and repopulated with 5×10^6 syngeneic bone marrow cells were tested 2 mo later. Although *C. parvum* pretreatment failed to evoke any adjuvant effect against SIII in these mice, no significance could be attached to the experiments in view of the inconsistent activity of *C. parvum* in the irradiated controls with an intact thymus (the latter animals had been previously shown to have persistent hyporesponsiveness to SIII (22).

More provocative observations were made with adult thymectomized, nonirradiated mice. Such animals did not differ from sham-operated controls on testing 21 days after operation. After 87 days, although the mean PFC response to SIII was $2\frac{1}{2}$ times higher in the thymectomized than in the control mice, this difference was not significant ($P = 0.2$). *C. parvum* was, however, ineffective in mice 87 days postthymectomy (Table 1). Consideration of the interpretation of this experiment will be deferred to the Discussion.

Possible macrophage involvement might depend on a change in antigen handling or the release of stimulatory factors. With regard to the first, a difference was found in the clearance from the circulation of ^{14}C-SIII. Whereas elimination down to 10%

TABLE 1

EFFECT OF *C. parvum* ON THE PFC RESPONSE TO SIII IN ADULT THYMECTOMIZED AND SHAM-OPERATED MICE

Time of testing after thymectomy (days)	*C. parvum* 4 days before SIII challenge	PFC per spleen 5 days after 5 μg SIII[a]	
		Sham-operated mice	Thymectomized mice
21	+	5.409 ± 0.145 (256,450)	5.379 ± 0.086 (239,330)
	−	4.869 ± 0.073 (73,960)	4.950 ± 0.053 (89,130)
87	+	4.845 ± 0.154 (70,000)	4.768 ± 0.153 (58,610)
	−	4.424 ± 0.191 (26,550)*	4.839 ± 0.080 (69,020)*

[a] Log_{10} ± SE with antilogs in parentheses (n = 5).
* Not significant (P = 0.2).

in 3 days was similar in both *C. parvum*-treated and normal mice, thereafter the former group sustained a higher circulating "tail" of antigen than did the controls (Fig. 6). This is attributable to exocytosis of indigestible antigen from phagocytosed reservoirs (13) which occurs at a greater rate when the macrophages are hyperactive (23), as is the case with *C. parvum*-treated animals. The persisting ^{14}C-SIII levels in the latter were reduced to near those in controls by 900-R whole-body irradiation prior to injection (Fig. 6).

Nonspecific stimulatory activity of macrophages was sought initially by measuring the response to SIII of spleen cells transferred to lethally irradiated mice which had been previously injected with *C. parvum*. It was argued that donor cells would

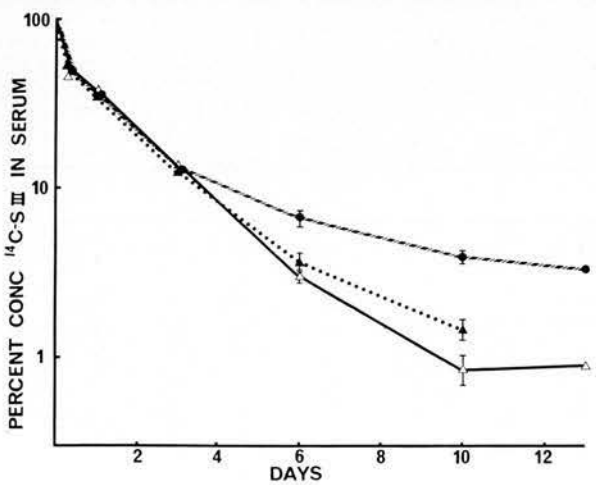

FIG. 6. Serum levels of ^{14}C-SIII after its injection iv (1 mg per 20 g body weight) in (a) normal mice (△——△), (b) mice injected with *C. parvum* 4 days previously (●----●), (c) as (b) but given 900 R immediately before injection (●-|-|-|-●). (Standard errors indicated, n = 8).

"home" to a spleen containing predominantly radioresistant *C. parvum*-activated macrophages. The unexpected outcome of this approach is described in the following section.

The Inhibitory Effect of C. parvum *on the B Cell Response to SIII in Lethally Irradiated Repopulated Mice*

A comparison was made of the PFC response to SIII in *C. parvum*-treated and normal mice which were irradiated with 900 R and repopulated with 8×10^7 syngeneic spleen cells (Table 2). A consistent inhibitory effect was found in the adjuvant-treated groups, in which PFC counts were suppressed to as low as <1% of normal. The following points emerge from Table 2: (1) the inhibitory effect was minimal in recipients which had been pretreated with smaller doses of *C. parvum*—10 and 2% of the standard 700 μg used throughout (Expt. A). (2) Suppression of the response was consistently greater when the SIII challenge was delayed 1 day, rather than given immediately after irradiation and repopulation (e.g., Expt. B). (3) The responses to optimal (5 μg) and suboptimal (0.5 μg) immunizing doses of SIII were similarly depressed. (The effect could not be reproduced by injecting a

TABLE 2

THE INHIBITORY EFFECT OF *C. parvum* INJECTED PRIOR TO LETHAL IRRADIATION OF RECIPIENT MICE ON THE RESPONSE TO SIII OF TRANSFERRED SYNGENEIC DONOR SPLEEN CELLS

Expt.	Dose of *C. parvum* (μg) 4 days before 900 R and 8×10^7 spleen cells	Interval between repopulation and SIII challenge (days)	Dose of SIII for challenge (μg)	PFC per spleen 5 days after SIII[a]		
				C. parvum mice	Controls (No *C. parvum*)	% Response in *C. parvum* mice
A	700			3.862 ± 0.079 (7,280)		19.7
	70	0	5	4.407 ± 0.059 (25,520)	4.568 ± 0.061 (36,980)	69.0
	14			4.460 ± 0.043 (28,840)		78.0
B		0		4.125 ± 0.128 (13,340)	5.130 ± 0.072 (134,900)	9.9
	700		5			
		1		3.063 ± 0.119 (1,555)	5.184 ± 0.032 (152,760)	0.75
C			0.5	2.765 ± 0.163 (582)	4.087 ± 0.140 (12,220)	4.8
	700	1				
			5	4.103 ± 0.109 (12,680)	5.105 ± 0.126 (127,350)	9.9

[a] Log_{10} ± SE with antilog in parentheses ($n = 5$).

TABLE 3

VARIATION IN THE EXTENT OF INHIBITION OF RESPONSE TO VARIOUS ANTIGENS PRODUCED BY *C. parvum* TREATMENT PRIOR TO LETHAL IRRADIATION AND REPOPULATION WITH SPLEEN CELLS

Antigen	PFC per spleen[a]		Serum antibody[b]	
	C.P. Mice[c]	Controls[d]	C.P. Mice[c]	Controls[d]
5 μg SIII	3.720 ± 0.110 (5,250)	4.879 ± 0.049 (75,680)	9.7 ± 0.6[e]	14.0 ± 0.3
10 μg Levan	3.546 ± 0.238 (3,515)	4.213 ± 0.062 (16,330)	7.3 ± 0.3[f]	8.3 ± 0.3
5×10^8 SRBC	4.405 ± 0.107 (25,410)	4.753 ± 0.036 (56,620)	6.5 ± 0.5[f]	9.0 ± 0.3

[a] Log_{10} ± SE with antilog in parentheses ($n = 5$).
[b] Log_2 ± SE.
[c] C.P. mice: *C. parvum* Day 0, 900 R, and 10^8 syngeneic spleen cells Day 4, antigen Day 5, and PFC assays Day 10.
[d] Controls: As above, without *C. parvum* pretreatment.
[e] Hemolytic antibody assayed.
[f] Hemagglutinating antibody assayed.

TABLE 4

LOSS OF INHIBITORY INFLUENCE ON SIII RESPONSE IN IRRADIATED *C. parvum*-TREATED MICE BY DELAYING INJECTION OF ANTIGEN OF REPOPULATING SPLEEN CELLS.

Experiment	Day of administration after 900 R		PFC per spleen 5 days after SIII[a]	
	8×10^7 spleen cells	SIII	C.P. mice[b]	Control
A Delay in SIII challenge[c]	0	1	3.282 ± 0.139 (1,910)	4.174 ± 0.091 (14,930)
	0	2	3.846 ± 0.205 (7,020)	4.060 ± 0.055 (11,480)
	0	3	4.264 ± 0.048 (18,370)[d]	4.111 ± 0.043 (12,910)[d]
	0	6	3.709 ± 0.033 (5,120)	3.820 ± 0.164 (6,610)
B Delay in spleen cell repopulation	1	2	4.723 ± 0.102 (52,840)	N.T.[e]
	3	4	4.739 ± 0.114 (54,820)	4.847 ± 0.055 (70,300)

[a] Log_{10} ± SE with antilog in parentheses ($n = 5$).
[b] C.P. mice = injected with 700 μg *C. parvum* iv 4 days before irradiation.
[c] SIII challenge = 0.5 μg (Expt A); 5 μg (Expt B).
[d] $P < 0.05$.
[e] Not tested.

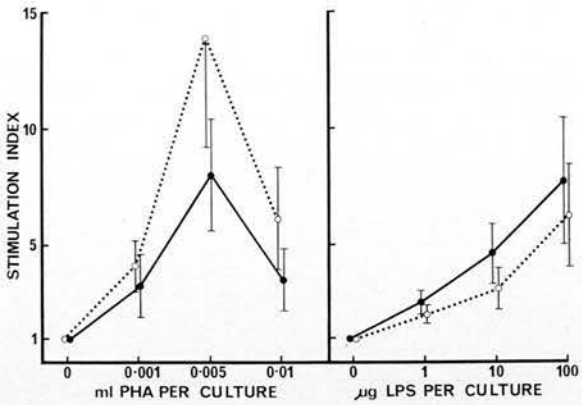

FIG. 7. Responsiveness to PHA and LPS of spleen cells removed from normal (●——●) and *C. parvum* pretreated (○····○) mice 1 day after they had been lethally irradiated (900 R) and repopulated with 10^8 syngeneic spleen cells. (Standard errors indicated, $n = 3$).

standard dose of *C. parvum* into irradiated normal mice repopulated with spleen cells. In this model only a minimal depression of the response to SIII was detected).

The immunosuppressive potential of *C. parvum* pretreatment in this model was assessed with two other antigens in parallel with SIII—the thymus-independent polysaccharide levan and sheep erythrocytes (Table 3). The PFC response to levan was 21% normal, although the hemagglutinin titer was reduced by only 1 $\log_2$ unit. The PFC response to SRBCs was 44%, while the hemagglutinin titer fell 2.5 units. The extent of inhibition is less striking with these antigens and was not explored in further detail.

The potent inhibitory influence on the SIII response in the *C. parvum*-treated radiation chimeras was found to be relatively transient in experiments where either spleen cell repopulation or antigen challenge were delayed (Table 4). No significant PFC suppression was found when the cells were injected 1 or 3 days postirradiation, instead of on the same day (Expt. B). The intense depression of response found in mice repopulated immediately after irradiation and challenged with SIII 24 hr later had largely disappeared when injection of antigen was made after 48 hr (Expt. A). A slight overshoot in the direction of stimulation was found after Day 3 challenge.

Rapid recovery of responsiveness to SIII was also found in a cell-transfer system. Lethally irradiated mice were injected with 7×10^7 spleen cells from donors which had been themselves irradiated with 900 R and repopulated with 7×10^7 spleen cells 1 day previously, with or without *C. parvum* pretreatment. No difference in PFC response to 5 μg SIII was found between the two groups.

Although it seems likely that suppression of SIII responsiveness in *C. parvum*-pretreated radiation chimeras would involve inhibition of cellular proliferation, no impairment in reactivity *in vitro* to the nonspecific mitogens PHA and LPS (acting on T and B cells, respectively) was apparent in spleens removed from *C. parvum*-treated mice 1 day after irradiation and repopulation (Fig. 7). These results are in keeping with the cell-transfer experiments and suggest that the suppression is entirely reversible by removing cells from an inhibitory environment, rather than a process involving lymphocyte damage.

The likelihood that the immunosuppressive influence of *C. parvum* described is

attributable to a radiation-induced effect on activated macrophages is considered in the Discussion.

DISCUSSION

The B cell response to the T-independent antigen SIII was considerably augmented by the adjuvant activity of *C. parvum*, when the upper range of immunogenic doses of the polysaccharide was used. No such effect was elicited by *B. pertussis*, which either diminished the PFC level when given together with SIII or did not change it when injected, like *C. parvum*, 4 days previously. The failure of *B. pertussis* to act as an adjuvant for a large dose of SIII contrasts with Dresser's finding (3) that it would augment the response of B cells to higher doses of sheep erythrocytes in T-deprived mice. Further evidence of the efficacy of *C. parvum* as a B cell adjuvant has recently come from studies on DNP coupled to levan, a T-independent carrier. The antihapten response, which requires both hapten and carrier-specific B cells (24) could be amplified by *C. parvum*, but not by *B. pertussis* or Freund's adjuvant (25). In the present experiments, the kinetics of the adjuvant effect, the inability to transfer it adoptively and the normal *in vitro* reactivity of spleen cells to the B cell mitogen LPS all suggest that *C. parvum*-induced amplification of the PFC response to SIII is due to some extrinsic control rather than to a direct effect on B lymphocytes. The most likely candidates to fit this role, T cells and macrophages, will be considered.

The first point to be made is that the SIII response can be amplified by T cell activity, both nonspecifically in the initial stage of GVH reaction (21) and specifically when SIII is coupled to a thymus-dependent carrier against which mice have been primed (26). The recent *in vitro* experiments of Basten and Feldmann (27) support the view that these effects are due to release of two distinct extracellular factors from T cells. Certain observations, however, argue against activation of thymus-derived lymphocytes as being an essential step in the adjuvant effect of *C. parvum* on the SIII response. First, no amplification was found using suboptimal doses of polysaccharide, the dose range for which a potential T cell-mediated activity would be expected to be most in evidence (e.g., 3). (The possible objection that small amounts of SIII would be deviated by intense macrophage activation after *C. parvum* seems improbable in view of (a) the clearance data presented on ^{14}C-SIII and (b) the stimulation produced against smaller doses of SIII by GVH reaction (21) where macrophage activation is even more pronounced (15). Second, a variety of T cell functions (PHA responsiveness, MLC and GVH reactivity) are inhibited, not stimulated, by the same *C. parvum* pretreatment which we have studied (10).

Unfortunately, our attempts to demonstrate an obligate role for T cells by means of deprived, irradiated mice were precluded by the inconsistent effect of *C. parvum* in irradiated repopulated controls, which respond poorly to SIII (see 22). The results with adult thymectomized, nonirradiated mice deserve comment, however, as they impinge on the debatable "suppressor" T cell. The conflicting evidence relating to this still hypothetical mechanism for regulation of the response to T-independent antigens is reviewed elsewhere (see 28), but two positive findings cannot be disregarded. (1) ALS treatment augments the SIII response (29) and the effect can be abrogated by T cell replacement (30). (2) Adult thymectomy is followed after a few weeks by heightened responses to PVP (31). A similar trend to the latter is recorded here with SIII, although the difference was not large enough

to be statistically significant with groups of five mice. As the response of such thymectomized mice was not increased further by adjuvant, it could be postulated that suppressor T cells (like other T cell activities) might be inhibited by *C. parvum*. This seems highly improbable, however, in view of the recent demonstration by Manning, Reed, and Jutila (32) that congenitally thymusless (nude) mice show PFC responses to SIII indistinguishable from those in siblings with intact thymuses.

An important role for macrophages in the cellular events underlying adjuvant activity was established (for some agents, at least) by the experiments of Unanue, Askonas, and Allison (33). Subsequent experiments by Askonas and Jaroskova (34) suggested that adjuvant-activated macrophages stimulated the differentiation and proliferation of antigen-triggered lymphocytes, either by membrane contact or by release of a factor. Activity of this type has recently been demonstrated *in vitro* in the supernatant fluid of macrophage cultures (35). The suggestion was made by Askonas and Jaroskova that the adjuvant-induced change in macrophages involved labilization of lysosomes.

There are strong grounds for believing that macrophage activation is the primary event behind the B cell-stimulating activity of *C. parvum*. (1) This bacterium is among the most potent known stimulants of the reticuloendothelial system (7), producing the appearance of large numbers of hyperphagocytic macrophages rich in lysosomal enzymes. The delayed onset of adjuvant activity produced by *C. parvum* is coincident with the development of this RE stimulation. (2) *C. parvum*-activated macrophages exert a direct inhibitory activity on T cells (11). These same phagocytes might induce the opposite effect in B cells if the latter have a much higher threshold for stimulation than T cells, as has been shown with *B. pertussis* (3). On this basis, one would predict that even higher levels of macrophage activity would inhibit B cells also. The stimulating factor in macrophage cultures studied by Hoffmann and Dutton (35) was strongly inhibitory to an *in vitro* immune response when used in higher dosage. In the present experiments a potent immunosuppressive effect was exerted on B cells transferred to *C. parvum*-pretreated (but not normal) irradiated recipients, which was a transient and wholly reversible phenomenon in which no intrinsic change or damage of lymphoid cells was implicated. The possibility that transferred cells were suppressed by an effective scaling-up in the level of "activity" emanating from stimulated macrophages after radiation damage is currently under investigation. Finally, it is worth noting that irradiated normal recipients repopulated with 8×10^7 spleen cells and immediately challenged with SIII have consistently shown PFC responses two to five times greater than intact, nonirradiated mice (unpublished data). These stimulatory and inhibitory influences might be attributable solely to quantitative differences in the same causal event.

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Cellular mechanisms underlying the adjuvant activity of *Corynebacterium parvum:* interactions of activated macrophages with T and B lymphocytes

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Abstract The effect of an intravenous injection of killed *Corynebacterium parvum* 4–7 days previously has been studied in mice with regard to various functions of T and B lymphocytes. The following T cell activities are inhibited: (1) Graft-versus-host reaction (by pretreatment of host or donor), (2) Mixed lymphocyte reactivity, and (3) PHA responsiveness of spleen or blood lymphocytes (but not lymph node cells). Removal of macrophages from the spleen cells of *C. parvum*-treated donors restores full responsiveness to PHA. Conversely, these cells (or their glass-adherent fraction) will inhibit normal cells.

Splenic B cells in *C. parvum*-treated mice react normally to the mitogen lipopolysaccharide. A strong adjuvant effect is demonstrable on the IgM response to higher immunizing doses of the T-independent antigen type 3 pneumococcal polysaccharide (SIII). By contrast, *Bordetella pertussis* exerts only an inhibitory influence on the response to SIII. The reactivity of spleen cells to SIII is transiently but potently suppressed when they are transferred into mice irradiated with 900 R and pretreated with *C. parvum*.

Both 19S and 7S components of the response to the T-dependent antigens, sheep and rat erythrocytes, are amplified by treatment with *C. parvum*. The former persists longer and can be elicited by otherwise sub-immunogenic doses, while the latter is most pronounced after higher doses. A comparison between thymus-deprived and intact mice implies that (1) *C. parvum*-treated deprived mice give 19S and 7S responses comparable to those of intact mice without adjuvant, and (2) *C. parvum* does not inhibit the normal cooperative function of T cells.

From these and other data we conclude that *C. parvum* inhibits T cell-mediated immunity and exerts a powerful adjuvant effect on B cells. It is argued that both stimulatory and suppressive influences are mediated by activated macrophages, which also seem likely to play a central role in determining concurrent increased resistance to tumours. Possible mechanisms whereby these macrophages might operate are discussed briefly.

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The striking stimulatory activity of *Corynebacterium parvum* on lymphoreticular tissues was originally discovered during a systematic search within this genus for species which might in this respect resemble certain of the related mycobacteria. In the event, *C. parvum* has turned out to be the most potent bacterial stimulant of the macrophage system currently available (Halpern *et al.* 1964). A single injection of killed organisms provokes a prolonged and intense hyperphagocytic response due to the emergence of very large numbers of highly activated macrophages. *C. parvum* also possesses a strong adjuvant property which is demonstrable either by substituting it for mycobacteria in complete Freund's adjuvant (Neveu *et al.* 1964) or by injecting it in saline a few days before, but not together with, antigen (Biozzi *et al.* 1966). (The second of these approaches is considered in this paper.) Another attribute which *C. parvum* shares with *Mycobacterium tuberculosis* (strain BCG) is the capacity to increase resistance to a wide range of experimental mouse tumours, but it has, unlike BCG, the additional advantage of being effective in the form of a killed vaccine (Woodruff & Boak 1966; Halpern *et al.* 1966; Currie & Bagshawe 1970; Smith & Scott 1972).

We describe here a series of experiments on the influence of pretreatment with *C. parvum* on the function of T and B lymphocytes, which we undertook for two reasons: (1) Preliminary studies suggested that modulation of the immune response by *C. parvum* had important qualitative as well as quantitative differences from the effect of other commonly used adjuvants. (2) We felt that more detailed information on this aspect might assist the delineation of the mechanisms by which *C. parvum* augments resistance to tumours. Unless stated otherwise, all experiments were performed in CBA strain mice. A standard suspension of killed *C. parvum* (strain CN6134) with a concentration of 7 mg/ml (Wellcome Research Laboratories) was used throughout, with the exception of earlier experiments (described in Table 1) which used the original Pasteur strain 936B.

EFFECTS ON T CELL FUNCTION

One of the earlier attempts to analyse the cellular mechanisms underlying these activities of *C. parvum* concerned a pronounced reduction in mortality rate which followed the induction of a graft-versus-host (GVH) reaction by parental lymphoid cells in pretreated adult F1 hybrid recipients (Table 1) (Howard *et al.* 1967). The effect was more striking than that obtained by analogous pretreatment with BCG (Biozzi *et al.* 1965) and from karyotypic analysis was clearly attributable to an inhibition of the proliferation of the donor cells (Table 1).

TABLE 1

Inhibition of GVH reaction induced by 10^8 parental strain spleen cells following pretreatment of F1 hybrid recipients with *C. parvum*

Recipients	*Strain combination: C57BL/6 → (C57BL/6 × C3H)F1*		*C57BL → (C57BL × CBA-T6T6)F1*	
	Mice dying/ no. injected	*Harmonic mean survival time (days)*	*% donor mitoses*	
			day 2	*day 12*
Untreated	24/25	21.8	18.0	88.4
C. parvum given 4 days before spleen cells	6/30	85.7	0.2	2.7

(Data from Howard *et al.* 1967.)

We suggested at the time, on the negative grounds of having excluded several alternatives experimentally, that amplification of allogeneic inhibition might be the causal mechanism. This view is no longer tenable in the light of more recent studies, to be described now, which have revealed the phenomenon as part of a more widespread depression of T cell function.

(1) Pretreatment of C57BL donors with *C. parvum* considerably weakens the GVH reactivity of their spleen cells in (CBA × C57BL)F1 recipients, as assessed by the relative spleen index (Table 2) (Scott 1972*a*). (2) A parallel depression was found in the mixed lymphocyte reactivity (MLR) of CBA spleen cells in a one-way system with lethally irradiated DBA/2 cells. Stimulation, as

TABLE 2

Reduced GVH reactivity of spleen cells from *C. parvum*-pretreated C57BL donors in (CBA × C57BL)F1 adult mice

Donor genotype	*Relative spleen weight (mg/10 g body wt.) ± S.E. of recipients (n = 16) 9 days after injection of 10^8 spleen cells from:*	
	Normal donors	*C. parvum-treated donors*
Parental (C57BL)	96.4 ± 5.9	61.8 ± 2.2
Host (F1)	39.4 ± 2.1	45.7 ± 1.9
Relative spleen index[a]	2.4	1.4

$$a = \frac{\text{Relative spleen weight with parental cells}}{\text{Relative spleen weight with F1 cells}}$$

(Data from Scott 1972*a*.)

judged by increased DNA synthesis in mixed compared with control cultures (containing irradiated syngeneic cells), was consistently reduced by treating the cell donors with *C. parvum* (Scott 1972*a*). (3) Scott (1972*a*) found that yet another T cell function, responsiveness to phytohaemagglutinin (PHA), is even more strikingly suppressed. Spleen cells, pooled lymph node cells and blood leucocytes were isolated seven days after the intravenous injection of 1.4 mg of *C. parvum* and tested for stimulation of DNA synthesis as measured by the

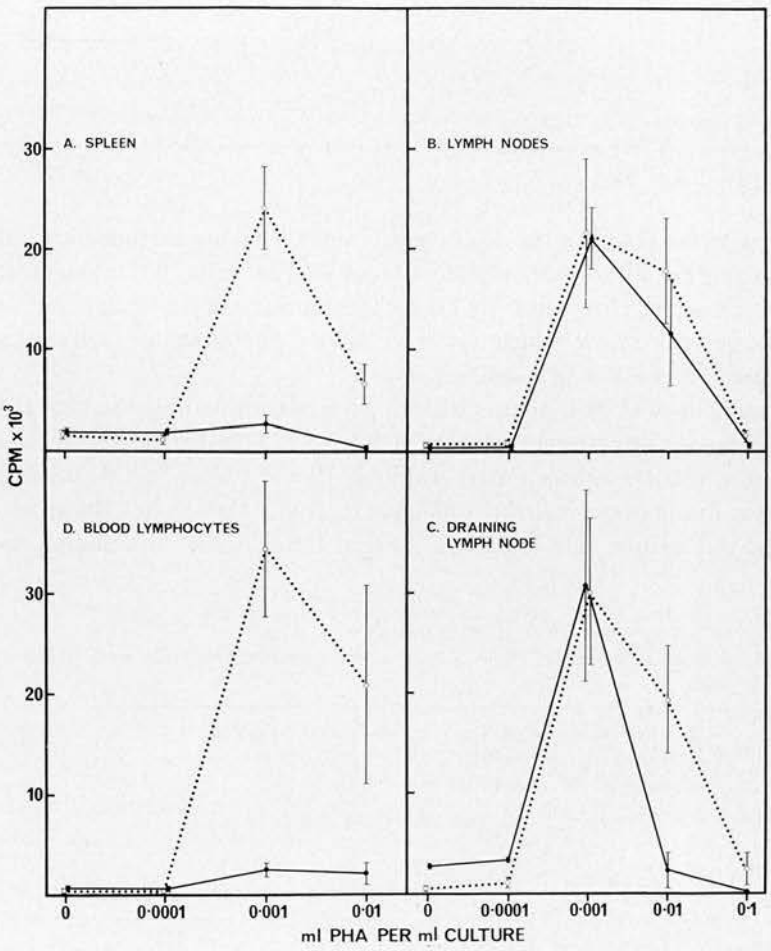

FIG. 1. PHA responsiveness of lymphocytes from mice injected with 1.4 mg *C. parvum* seven days previously (●—●), compared with normal mice (○ · · · ○). The injection was intravenous in the case of spleen cells (A), pooled lymph node cells (B) and blood lymphocytes (D). Draining popliteal lymph node cells were assayed after footpad injection. Standard errors shown; $n = > 5$. (From Scott 1972*a* with permission of *Cellular Immunology*.)

incorporation of [^{14}C]thymidine. The responsiveness of T cells in spleen and blood to the entire range of stimulating doses of PHA was totally suppressed, whereas the responsiveness of lymph node cell suspensions was unaffected (Fig. 1 A, B and D). Inhibition of reactivity to supra-optimal doses of PHA was found in cells of the draining popliteal lymph node, however, when *C. parvum* had been injected into the footpads (Fig. 1C). The [^{14}C]thymidine pulse was routinely added after 48 hours of culture with PHA *in vitro*, as the possibility of a premature response by cells from *C. parvum*-treated donors was excluded. Analogous treatment with another member of the same genus, *C. diphtheriae*, which is devoid of macrophage-stimulating and adjuvant activity, failed to modify responsiveness to PHA.

This refractoriness of spleen cells from mice treated with *C. parvum* has proved to be reversible (Scott 1972*b*). Normal responsiveness to PHA reappeared after the removal of glass-adherent cells or, more specifically, of macrophages by magnetic elimination of cells which take up carbonyl iron (Fig. 2). Conversely, the PHA reactivity of normal spleen or lymph node cells was inhibited

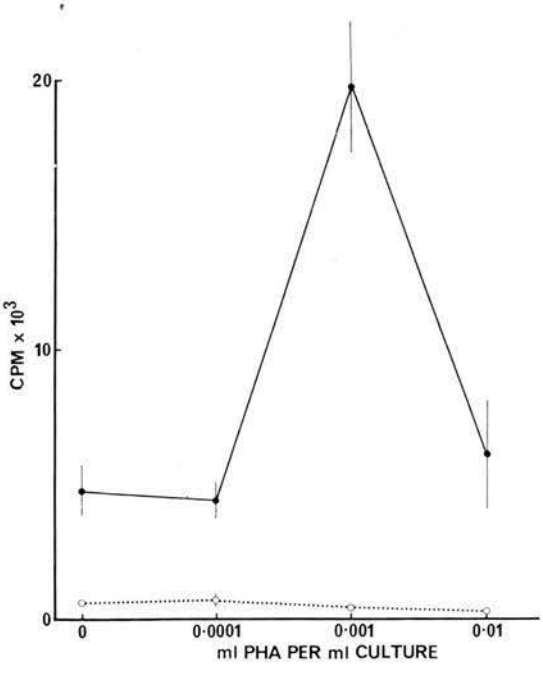

FIG. 2. Recovery of responsiveness to PHA by spleen cells from donors pretreated with *C. parvum*, after the removal of macrophages by magnetic separation. Total cells (○····○); macrophage-depleted cells (●—●). Standard errors shown; $n = 3$. (From Scott 1972*b* with permission of *Cellular Immunology*.)

when they were mixed with spleen cells (or their glass-adherent fraction) from *C. parvum*-treated donors.

These findings imply that the functional depression of T cells after the injection of *C. parvum* is attributable to the influence of activated macrophages. The normal response to PHA of lymph node cells after intravenous injection of the bacteria would merely reflect their failure to localize in the nodes when administered by this route.

Impressive evidence has recently been obtained of the suppression by *C. parvum* of T cell-mediated immunity *in vivo*. The development of contact sensitivity in mice to picryl chloride or oxazolone was found by Asherson & Allwood (1971) to be depressed by prior intravenous injection of *C. parvum*. J. E. Castro (unpublished results) has obtained a prolongation of up to 3–4 days in the mean survival time of skin allografts across an H–2 barrier in mice (A→CBA) by injecting *C. parvum* one week before or on the day of grafting. All these *in vitro* and *in vivo* observations are consistent with the conclusion that, with regard to cell-mediated immunity, T lymphocyte function is depressed, not stimulated, by prior treatment with *C. parvum* in saline. Although the experiments with PHA indicate that this is an indirect effect under the influence of activated macrophages, it remains to be proved formally that this is a general control mechanism extending to all the T cell functions described. We shall discuss later how the macrophage might fulfil this role, after the function of B cells and the cooperative function of T cells in *C. parvum*-treated mice have been considered.

It must be stressed that all these examples of depressed T cell function follow the systemic administration of *C. parvum*. When injected locally as the bacterial component of complete Freund's adjuvant, it potentiates the development of delayed hypersensitivity (Neveu *et al.* 1964).

EFFECTS ON B CELL FUNCTION

The suppressed reactivity to PHA of T cells in *C. parvum*-treated mice is not paralleled by the homologous response of their B cells to the mitogen *E. coli* lipopolysaccharide (LPS). The extent to which spleen cells from treated and normal mice are stimulated *in vitro* by various doses of LPS is similar (Howard *et al.* 1973).

We have found that pretreatment with *C. parvum* exerts a strong adjuvant effect on the B cell response to the thymus-independent antigen type 3 pneumococcal polysaccharide (SIII) (Howard *et al.* 1973). Immunity to SIII is exclusively humoral and is strictly independent of T cell cooperative function

(Howard *et al.* 1971), although it *can* be amplified by T cell activity during the early phase of the GVH reaction (Byfield *et al.* 1973). Prior injection of *C. parvum* did not increase the counts of direct plaque-forming cells in the spleen after small immunizing doses of SIII (0.1–0.5 μg), but produced an 8– to 10–fold rise with higher doses (2–50 μg) (Fig. 3). The induction of tolerance by 500 μg of SIII was not impaired. The time course of the response to an optimal immunizing dose of 5 μg (Fig. 4) indicates that the initial rate of expansion of PFC was not increased and suggests that the number of SIII-reactive B cells had not been augmented by the adjuvant. Rather, *C. parvum* pretreatment appeared to sustain the response at a higher level. It was obligatory to inject *C. parvum* several days before SIII; simultaneous administration significantly reduced the PFC response. A comparison was made with the effect of *Bordetella pertussis*, in view of the fact that its adjuvant activity is predominantly T cell orientated, except with very high doses of antigen (Allison & Davies 1971; Dresser 1972). Injection of 2×10^9 *B. pertussis* organisms four days before 5 μg SIII was without effect, while their administration together with this antigen was highly suppressive; in one experiment the response fell to 10% of normal. This is noteworthy, because injection of the same batch of *B. pertussis* with sheep erythrocytes revealed its customary immunostimulatory effect.

Our colleague P. del Guercio (1972) has recently found similar evidence of the efficacy of *C. parvum* for stimulating B cell responses during his studies on

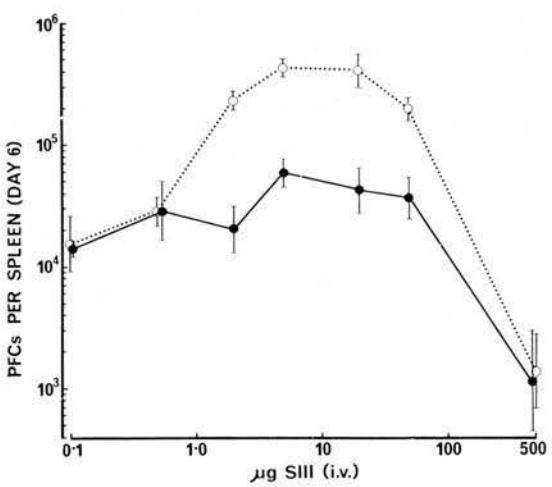

FIG. 3. Adjuvant effect of injecting 700 μg *C. parvum* four days before various doses of SIII. Response measured by counts of direct plaque-forming cells (PFC) after six days. *C. parvum*-treated mice (○····○); normal mice (●—●). Standard errors shown; $n = 5$. (From Howard *et al.* 1973 with permission of *Cellular Immunology*.)

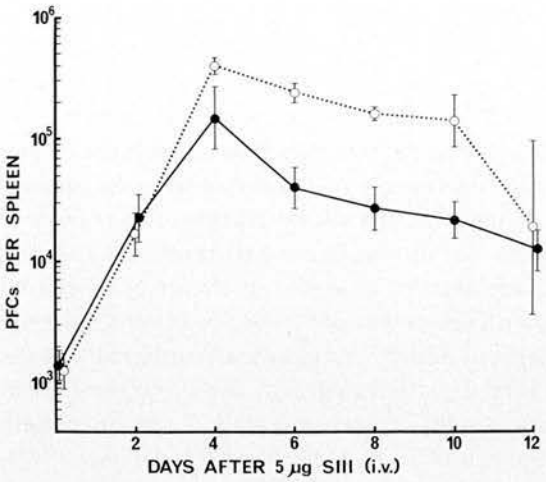

FIG. 4. Effect of *C. parvum* pretreatment on the course of the splenic PFC response to 5 μg SIII. Conventions as in Fig. 3. (From Howard *et al.* 1973 with permission of *Cellular Immunology*.)

another thymus-independent antigen, dinitrophenol coupled to levan (DNP–LE)—a complex which appears to involve both DNP- and LE-specific B cells (del Guercio & Leuchars 1972). Whereas pretreatment with *C. parvum* effectively augments the anti-hapten response to DNP–LE, both Freund's adjuvant and *B. pertussis* are ineffective. The failure of these other agents to display their adjuvant property with T-independent antigens may be a reflection of their predominant stimulatory influence on T cells. Our own efforts to implicate T cells in the effect of *C. parvum* on the anti-SIII response by studying thymus-deprived mice have been thwarted so far by the inconsistent behaviour of irradiated controls, which remain chronically hyporesponsive to this antigen.

The suggestion from Fig. 4 that *C. parvum* does not produce an intrinsic change in splenic B lymphocytes was borne out by the absence of any adjuvant activity with regard to SIII after the transfer of spleen cells into irradiated recipients. We consider that the extrinsic influence most likely to explain the augmented response to T-independent antigens is the activated macrophage, for the following circumstantial reasons: (1) *C. parvum* is phagocytosed within a very short time of being injected into the circulation; (2) the lag period before the onset of a demonstrable immunopotentiating effect coincides with the emergence of activated macrophages; and (3) macrophage function influences the activity of T cells in *C. parvum*-treated mice. We sought evidence of this postulated stimulatory activity by measuring the anti-SIII response of spleen cells transferred into lethally irradiated mice that had been previously injected

with *C. parvum*, on the ground that donor cells would 'home' to a spleen consisting predominantly of radioresistant activated macrophages. The result of this approach was dramatically converse to expectation, for the response was profoundly suppressed in *C. parvum*-pretreated, irradiated recipients of normal spleen cells when SIII was injected up to 24 hours after repopulation (Table 3).

TABLE 3

The inhibitory effect of 700 μg *C. parvum* injected before lethal irradiation of recipient CBA mice on the response to SIII of transferred syngeneic donor spleen cells

SIII challenge (μg)	*Interval between repopulation and challenge (days)*	*PFCs per spleen (day 5)*		*% response in treated mice*
		Treated mice	*Controls*	
5	0	13 340	134 900	9.9
	1	1560	152 800	0.75
0.5	1	580	12 220	4.8
	2	7020	11 480	61.1
	3	18 370	12 910	142

All mice irradiated with 900R and repopulated with 8×10^7 syngeneic spleen cells. Treated mice were injected with 700 μg *C. parvum* four days before irradiation. Geometric means of five animals shown. (Data from Howard *et al.* 1973.)

This effect was entirely reversible and disappeared when challenge was delayed until 48 hours or if the cells were transferred to secondary recipients. In parallel with this suppression of the anti-SIII response in *C. parvum*-treated mice, normal irradiated recipients generally showed PFC levels somewhat higher than those in intact mice. We considered the possibility that both suppression and stimulation might result from some damaging effect of lethal irradiation on activated and normal macrophages respectively. To test this hypothesis, we have recently compared the effect of crystalline silica (kindly provided by Dr A. C. Allison), which selectively damages macrophages. The same dual effect has been obtained. Injection of silica together with SIII depresses the response in *C. parvum*-treated mice while augmenting it in normal animals. The possible nature of what seem likely to be macrophage-determined effects will be discussed later.

EFFECTS ON THE RESPONSE TO THYMUS-DEPENDENT ANTIGENS

In view of the inhibitory influence of *C. parvum* treatment on various func-

tions of T cells associated with cell-mediated immunity, the question arises of whether or not their 'helper' role is affected similarly, in which case adjuvant activity might involve predominantly 19S at the expense of the more highly T-dependent 7S antibody. We have recently looked at the effect of *C. parvum* on responses to sheep and rat erythrocytes, estimating the total and 2-mercapto-ethanol (2ME)-resistant haemagglutinin titres after a wide range of doses of both antigens (Figs. 5 and 6). Direct and indirect PFC counts in the spleen were also

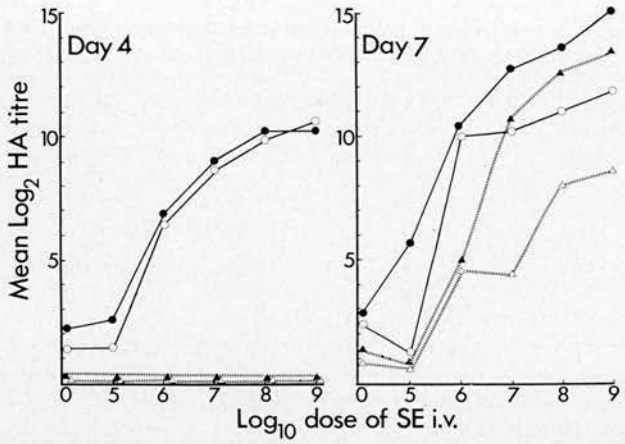

FIG. 5. Adjuvant effect of injecting 700 μg *C. parvum* four days before various doses of sheep erythrocytes (SE). Mean antibody titres ($n = 5$) on days 4 and 7. Total antibody: *C. parvum*-treated mice (●—●); controls (○—○). 2-Mercaptoethanol-resistant antibody: *C. parvum*-treated mice (▲ ıı ▲), controls (△ ıı △).

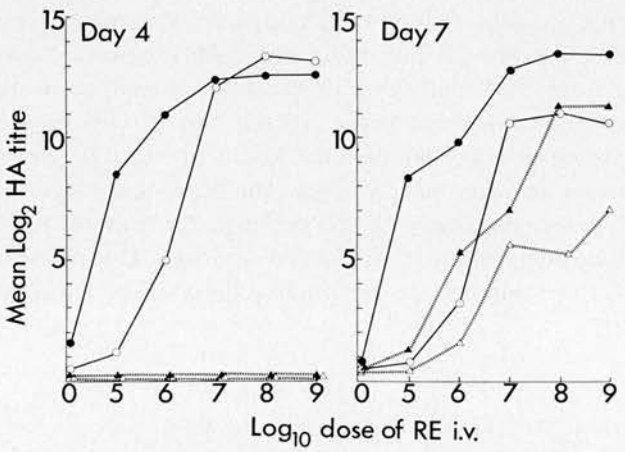

FIG. 6. Adjuvant effect of *C. parvum* pretreatment on the response to various doses of rat erythrocytes (RE). Experimental design and conventions as in Fig. 5.

TABLE 4

The effect of pretreatment with 700 μg *C. parvum* on the response to various doses of sheep erythrocytes in terms of direct and indirect splenic PFCs

Dose of sheep red cells (i.v.)	*C. parvum treatment (4 days before challenge)*	*PFCs per spleen*[a]		
		Direct		*Indirect*
		Day 4	*Day 7*	*Day 7*
10^8	+	1 739 000	940 600	5 506 000
	−	877 400	25 500	535 400
10^6	+	52 400	328 000	44 560
	−	40 700	35 000	1450
10^5	+	9850	19 700	0
	−	600	250	0

[a] Geometric means of five mice.
(Data of G. H. Christie & J. G. Howard, unpublished.)

assayed in the mice injected with sheep erythrocytes (Table 4). Indirect PFC counts and 2ME-resistant antibody titres were taken as measures of a 7S antibody response. Pretreatment with *C. parvum* amplified 19S (2ME-sensitive) antibody formation in response to lower doses of both antigens, so that a response was detectable after only 10^5 rat or sheep erythrocytes, which failed to stimulate normal mice. The data in Table 4 also show that the IgM response (direct PFCs) after higher doses is more sustained in *C. parvum*-treated mice, remaining elevated at day 7 when indirect PFCs are already numerous. A pronounced augmentation of the 7S response to higher doses of both antigens was detectable serologically and, for sheep erythrocytes, by indirect PFCs.

As *C. parvum* appears to exert such a potent adjuvant effect on B cells, we investigated whether its ability to amplify 7S responses might involve a T cell by-pass mechanism. Thymectomized and normal mice were irradiated with 900R, repopulated with 5×10^6 bone marrow cells and immunized with 10^8 sheep red cells 20 weeks later (Table 5). The thymus-deprived group gave small direct PFC and no indirect PFC responses by day 7, whereas when *C. parvum* was administered to them four days before challenge they developed direct and indirect PFC levels of similar magnitude to those found in immunized control mice. On the other hand, the considerably greater responses in the control recipients of *C. parvum* implies that the normal cooperative activity of T cells is not impaired by the adjuvant. Titrations of 2ME-resistant antibody also indicated a similar increase in the 7S category as did the indirect PFCs in this experiment. Is the considerable amplifying effect of *C. parvum* on 7S as well as 19S antibody production in T-deprived mice due to its potent ability to stimulate B cells? Although the development of a 7S response in these mice is particularly strik-

TABLE 5

A comparison of the adjuvant effect of 700 μg *C. parvum* on the response to sheep erythrocytes in thymectomized and intact bone marrow-reconstituted mice

Recipients[a]	*C. parvum treatment (4 days before challenge)*	*Response 7 days after 10^8 sheep red cells (i.v.)*[b]			
		PFCs/spleen		*Haemagglutinin (log_2)*	
		Direct	*Indirect*	*Total*	*2-ME resistant*
Thymectomized	+	37 960	133 500	9.5	5.8
	—	6980	210	7.0	< 1
Controls	+	630 400	3 427 000	14.6	12.2
	—	67 800	74 360	9.4	7.2

[a] 900R irradiation and repopulation with an intravenous injection of 5×10^6 bone marrow cells, with or without prior thymectomy.
[b] Geometric means of five mice.
(Data of G. H. Christie & J. G. Howard, unpublished.)

ing, it is not unique. Dresser (1972) found that T-deprived mice would develop 7S responses to sheep red cells (although only with a very high immunizing dose) under the influence of *B. pertussis*. Chiller & Weigle (1973) have also shown clearly that the B cell mitogen LPS can stimulate mice to give a 7S response to human gammaglobulin when their T cells are tolerant of this antigen. The standard thymectomized irradiated, marrow-repopulated mouse does, however, retain a few T cells. To exclude the possibility that *C. parvum* might operate by some functional expansion of these, we have also examined its effect in congenitally thymus-less (nude) mice. Unfortunately, the adjuvant has proved to be toxic in these animals, so that we have not yet obtained a decisive answer by this approach.

EFFECTS ON T AND B CELLS IN RELATION TO ANTI-TUMOUR ACTIVITY

In the face of the cumulative evidence of suppression of relevant T cell activity, it seems highly unlikely that an increase of cell-mediated immunity could determine the augmented resistance to tumours induced by *C. parvum*. Direct support for this contention is provided by recent observations that this property of inducing tumour resistance is fully effective in mice deprived of T cells by thymectomy or by treatment with antilymphocyte serum (J. E. Castro, personal communication; Woodruff & Dunbar, this volume, pp. 287–300). Amplification of the humoral response or some component of it remains a possible, if unlikely, basis for a phenomenon which affects a diverse range of neoplasms. A recent experiment performed by Biozzi *et al.* (1972) seems to

argue against an exclusive determinant role for antibody. They have studied two lines of mice (Ab/H and Ab/L), genetically selected for high and low antibody responses, which can be demonstrated with any (including histocompatibility) antigen and involve all classes of immunoglobulins. A wide separation in antibody levels is still present after the injection of *C. parvum*. F1 hybrids between strains AKR and Ab/H or Ab/L retain respectively high and low responsiveness but, significantly, *C. parvum* induces resistance to the AKR lymphoma even more effectively in (AKR × Ab/L)F1 than it does in (AKR × Ab/H)F1.

These negative considerations strengthen the suspicion that in tumour resistance too it is the intensely activated macrophage which is the principal mediator of *C. parvum*-induced activity. Adequate evidence is now available of the cytotoxic capacity of macrophages against tumour cells in both specific and non-specific *in vitro* models (see Evans & Alexander 1972; Hibbs *et al.* 1972). Furthermore, the cytotoxic activity of macrophages against fibroblast target cells has been correlated with their degree of activation (McLaughlin *et al.* 1972). Our colleagues R. Bomford & M. Olivotto (personal communication) have recently noted that *C. parvum*-activated macrophages are considerably more effective than their normal counterparts in inhibiting the proliferation *in vitro* of a radiation-induced CBA leukaemia. Further elaboration of this argument is beyond the intended scope of this contribution.

REGULATORY INFLUENCE OF C. PARVUM-ACTIVATED MACROPHAGES

Our hypothesis, albeit based on an incomplete framework of data, is that the highly activated macrophages which appear in response to *C. parvum* occupy a central position as mediators of the described effects on T and B lymphocytes and on tumour cell growth. Many further obvious experiments need to be done before we can assess the general validity of this contention. How might these cells exert such an influence? One important factor with regard to adjuvant activity may be an improved or prolonged presentation of antigen. Frost & Lance (this volume, pp. 29–38) have found that *C. parvum* is one of the strongest inducers of 'lymphocyte trapping', which their evidence implies is a macrophage-mediated effect. This mechanism would facilitate extended contact between antigen-charged phagocytes and lymphoid cells and could provide a plausible explanation for the ability of *C. parvum* to stimulate a primary response to otherwise sub-immunogenic doses of sheep and rat erythrocytes. On the other hand, our finding that an adjuvant effect on the anti-SIII response occurs only with *higher* immunizing doses of polysaccharide suggests the additional operation of another mechanism.

Macrophages are a mandatory requirement for the induction of an immune response to many, although not all, antigens and seem to be essential for lymphocyte differentiation and proliferation *in vitro* (e.g. Sjöberg *et al.* 1972). There is compelling evidence that, after taking up *B. pertussis*, macrophages present a magnified proliferative stimulus to antigen-triggered lymphocytes (Unanue *et al.* 1969; Askonas & Jarošková 1970; Allison, this volume, pp. 73–94). With regard to *C. parvum*, this concept is wholly compatible with the data on B cell function, but why should some T cell activities be suppressed? One explanation would be quantitative, that the macrophage 'influence' is stimulatory or inhibitory according to dosage and that the effective thresholds for T cells (and perhaps tumour cells also) are lower than those for B cells. Hoffmann & Dutton (1971) described a factor released in macrophage cultures which would substitute for macrophages in stimulating *in vitro* responses to sheep erythrocytes, but was highly inhibitory when used in higher concentration. The conversion by irradiation or silica of the stimulatory effect of *C. parvum* on the anti-SIII response into inhibition may be due to a scaling-up in the release of some such factor. Alternatively, these opposing effects may be determined by different macrophage activities which might involve either membrane contact or release of factor(s). Although the experiments of Hoffmann & Dutton suggest the latter, no evidence has been obtained so far for an extracellular factor in *C. parvum*-induced suppression of T cell reactivity to PHA or proliferation of R1 leukaemia cells *in vitro* (Scott 1972*b*; R. Bomford & M. Olivotto, personal communication). Current understanding of the regulatory influence of macrophages over lymphocyte function is highly fragmentary and awaits further study, for which activation by *C. parvum* seems to provide a valuable approach.

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Depression of Delayed-Type Hypersensitivity by *Corynebacterium parvum*: Mandatory Role of the Spleen

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Mice pretreated with iv *Corynebacterium parvum* showed markedly reduced DTH reactivity to subsequently injected SRBC without concomitantly increased antibody levels. No DTH depression occurred if *C. parvum* was given at the same time, or after, antigen. Neither antigen sensitization at the draining lymph node level nor the subsequent loss of sensitized cells from the node was impaired by iv *C. parvum* pretreatment. Splenectomy before *C. parvum* completely abolished its depressive effect, and lymph nodes, even when directly stimulated by local injection of *C. parvum*, were unable to substitute for the spleen in DTH depression. Increased uptake of sensitized cells by the *C. parvum* stimulated spleen has been demonstrated, and the discrepancy between the spleen and lymph node performance is discussed in terms of the depression of T-cell activity by *C. parvum*-activated macrophages described previously.

INTRODUCTION

The anti-tumor activity of the reticuloendothelial stimulant (1, 2) and adjuvant (3, 4) *Corynebacterium parvum* has been demonstrated in both mice (5, 6) and humans (7). As an adjuvant, it appears unique in its ability to increase B-cell proliferation without the cooperation of T cells (4, 8). Other adjuvants, e.g., Freund's complete adjuvant (9) and *Bordetella pertussis* (4, 9, 10), require T cells to manifest their adjuvant properties at least where low antigen doses are concerned (11). That *C. parvum* actually depresses T-cell activity was first suggested from *in vitro* experiments showing the PHA responsiveness of lymphoid cells from *C. parvum*-treated mice to be depressed (12). This effect was attributable to inhibition of potentially reactive T cells by *C. parvum*-activated macrophages (13). Subsequent reports describing *C. parvum* depression of *in vivo* development of contact sensitivity to picryl chloride in mice (14, 22), along with the present report of impaired expression of DTH to sheep red cells (SRBC), provide *in vivo* evidence in support of the *in vitro* finding of T-cell inhibition by this adjuvant. In addition to its role in T-cell depression, the *C. parvum*-activated macrophage is now also reasoned to be the cell type instrumental in other *C. parvum*-mediated effects—namely, adjuvant and antitumor activity (8).

Tumor immunity is considered to be largely attributable to cell-mediated mechanisms, and, in light of the apparent T-cell depression by *C. Parvum*, the use of this agent in tumor therapy may seem anomalous. Indeed, under certain experimental conditions, *C. parvum* has been shown to impair active immunity to irradiated tumor

cells (5). The following work was therefore initiated to study further the nature of depression of T-cell reactivity at the whole animal level.

MATERIALS AND METHODS

Animals

Specific pathogen-free outbred CD-1 mice (Charles River Farms, Wilmington, MA) were used throughout except in experiments with thymectomized repopulated mice when (C57BL/6 × DBA/2)F_1 – (B6D2F_1) were substituted. Similar results were obtained with both strains.

T-Cell-Deprived Mice

Four- to 6 wk-old B6D2F_1 mice were thymectomized and 1 wk later given 900 R of gamma irradiation from a ^{137}Ce source. Terramycin (125 μg/ml) was given in the drinking water. The same or following day they received 5 × 10^6 syngeneic bone marrow cells iv. The mice were used 5–6 wk after reconstitution and 1 wk after antibiotic treatment had been discontinued.

Corynebacterium parvum

C. parvum (batch number EZ 174, 7 mg/ml, Burroughs Wellcome) was kindly donated by Dr. C. Adlam, and 0.1 ml (700 μg) was injected either iv or subcutaneously (sc) as described.

Splenectomy

Surgical splenectomy was performed under ether anesthetic and controlled for by sham splenectomy.

Delayed-Type Hypersensitivity (DTH) to Sheep Red Blood Cells (SRBC)

SRBC in Alsevers solution were purchased from the Animal Blood Center (P.O. Box 2, Syracuse, NY). Mice were sensitized by the injection of SRBC suspensions in saline either sc into the neck region (0.1 ml) or sc into the footpad (0.05 ml using a 30-gauge needle). Delayed-type hypersensitivity reactivity was elicited at various times after sensitization by sc injection of 10^8 SRBC into a previously uninjected hind footpad, and footpad swelling was measured 24 hr later with a Schnelltaster dial gauge caliper A02T (H. C. Kroplin, Schluchtern, Hassen, West Germany). Injection of sc SRBC caused no 3-hr footpad swelling (Arthus reactivity), and iv SRBC were used to study this phenomenon as described in the test. Footpad thicknesses are recorded as the difference between the antigen-injected footpad and the contralateral foot in units of 0.1 mm. The successful use of SRBC suspensions in DTH sensitization of mice has been described previously (15, 16) and conforms to all the established criteria by which DTH is recognized, including cell transfer and sensitivity to anti-θ serum (16).

Assay for Lymph Node DNA Synthetic Activity

At each time point indicated, five mice were injected iv with 20 μCi of tritiated thymidine (New England Nuclear) diluted in 0.9 % saline. Thirty minutes later

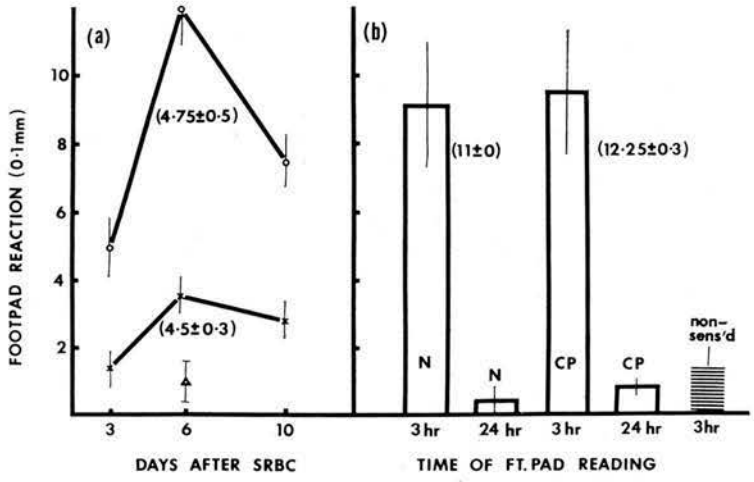

FIG. 1. (a) Kinetics of DTH (24-hr swelling) to SRBC elicited by footpad injection of 10^8 SRBC at various times after sensitization with 2×10^7 SRBC sc in either normal mice (○–○) or mice that had received *C. parvum* iv (700 μg) 7 days prior to sensitization (×–×). Figures in brackets are the reciprocal $\log_2$ hemagglutination titers ± standard error. Vertical bars represent ± standard error (n=5). (b) Arthus reactivity (3-hr swelling) elicited, as above, 6 days after sensitization with 2×10^7 SRBC iv in either normal mice (N) or mice that had received *C. parvum* iv (700 μg) 7 days prior to sensitization (CP). Normal non-SRBC-sensitized controls are included (horizontal bars). Vertical bars and figures in brackets are as for (a).

the popliteal nodes were removed and homogenized individually in 5% TCA. They were further extracted for DNA as described previously (17), and the uptake of tritiated thymidine was counted in a Beckman LS100 liquid scintillation counter.

Hemagglutination Assay

Microtitrations were performed in V-shaped plastic wells by serially diluting sera with saline. To 0.1 ml of diluted serum was added 0.1 ml of 1% SRBC suspension. All tests were incubated for 30 min at 37°C and then overnight in the refrigerator. Titers were recorded as $\log_2$ of the reciprocal of the last dilution showing agglutination.

EXPERIMENTAL

Effect of C. parvum Treatment on DTH and Arthus Reactivity

Mice were injected with *C. parvum* iv and 7 days later received a 2×10^7 SRBC sc in the back of the neck. Control mice received only sc SRBC. Delayed-type hypersensitivity reactions were elicited in the sensitized mice 3, 6, or 10 days after sensitization by injection of 10^8 SRBC into a hind footpad and comparing the swelling at 24 hr with that of the contralateral (Fig. 1a). Control mice developed considerable DTH, which peaked at Day 6, whereas *C. parvum*-treated mice showed markedly reduced responsiveness at all three test periods. The remaining DTH reactivity was, however, significant at both Days 6 and 10. Serum hemagglutinin titers at Day 6 in the two groups were not significantly different (Fig. 1a). No significant Arthus reactivity developed in mice sensitized with sc SRBC, so, to

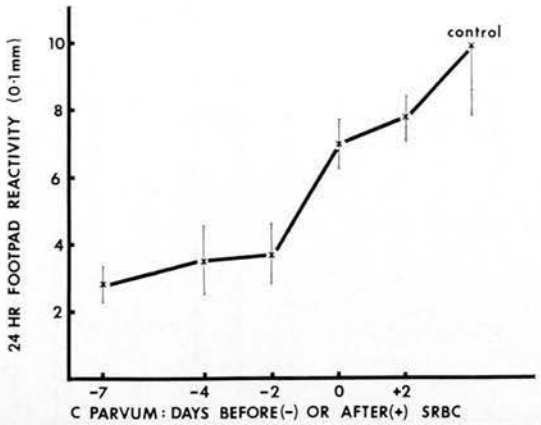

FIG. 2. The effect of *C. parvum* on DTH to SRBC when given at various times relative to SRBC sensitization. *C. parvum* (700 μg) was injected iv either before (−) or after (+) sensitization with 10^8 SRBC sc into the neck. DTH was elicited by footpad injection of 10^8 SRBC 4 days after sensitization. Control animals received no *C. parvum*. Vertical bars represent ± standard error (n=5).

examine the effect of *C. parvum* pretreatment on Arthus reactivity, similar groups of mice were sensitized with 2×10^7 SRBC iv. Footpad challenge was 6 days later, and swelling was measured at 3 and 24 hr (Fig. 1b). Arthus reactivities (3 hr) in control and *C. parvum*-treated mice were similar despite higher antibody levels in the latter group. Neither group developed DTH following this dose of iv SRBC.

Relationship between Time of C. parvum and Antigen Injection

C. parvum was injected iv into mice at the times shown in Fig. 2, relative to a sensitizing injection of 10^8 SRBC sc into the neck. Delayed-type hypersensitivity was elicited by footpad challenge 4 days after sensitization and measured 24 hr later. *C. parvum* injected iv 7, 4, and 2 days prior to SRBC sensitization was equally suppressive (DTH about 30% normal). However, there was no statistically significant impairment of reactivity when *C. parvum* was given either at the same time as, or 2 days later than, antigen (Fig. 2). It may have been that DTH was not depressed when *C. parvum* was injected 2 days after antigen merely because it had been active in the body only 2 days before DTH elicitation, whereas in the pretreatment groups it had been active for a longer period. To control for this, further experiments in which mice received *C. parvum* at Day +2 (after sensitization) and were challenged in the footpad at Day +8 (i.e., 6 days after *C. parvum* and comparable to those which received *C. parvum* at Day −2 and were challenged at Day +4) were performed, and again no significant DTH depression was found. That the degree of DTH inhibition is unrelated to the time that *C. parvum* has been acting in the animal is further evidenced by the fact that *C. parvum* at Days −7, −4, and −2 were equally suppressive rather than giving graded responses.

The Effect of C. parvum Pretreatment on the Lymph Node Proliferative Response to Antigen in Normal and T-Cell-Deprived Mice

To determine whether depressed DTH reactivity resulted from impaired antigen sensitization, the proliferative response in the lymph node draining the site of

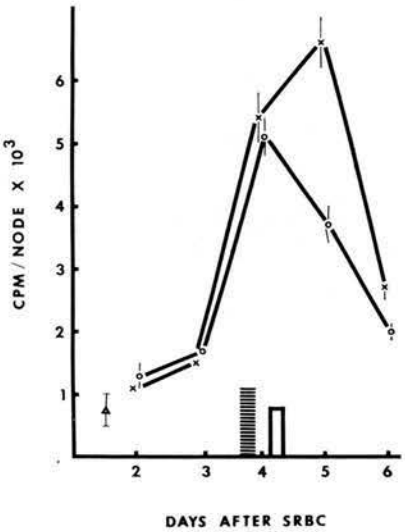

FIG. 3. Kinetics of DNA synthesis in the popliteal node in response to a hind footpad injection of 10^8 SRBC in either normal mice (○–○) or mice that had received *C. parvum* (700 μg) iv 7 days previously (×–×). Histograms: DNA synthesis, as above, 4 days after SRBC injection in T-cell-deprived mice that received *C. parvum* (700 μg) iv 7 days previously (open) or no pretreatment (horizontal bars). Background isotope incorporation by non-SRBC-stimulated nodes of normal mice is shown (△). Vertical bars represent ± standard error (n=5).

antigen stimulation was compared in normal and *C. parvum*-treated mice (Fig. 3). Instead of injecting the sensitizing SRBC into the neck as before, 10^8 SRBC in 0.05 ml of saline were injected sc into a hind footpad, and the incorporation of tritiated thymidine in the popliteal node was assessed at the days indicated. Previous experiments had determined that footpad sensitization was equally as effective at producing DTH as injections into the neck and also that it was susceptible to depression by *C. parvum* pretreatment. There was no impairment of the proliferative response of the lymph nodes in *C. parvum*-treated mice, there being, in fact, an overall increase in responsiveness due to a prolongation of the response. The levels of lymph node DNA synthesis in both normal and *C. parvum* mice 2 days after SRBC were not significantly different (Fig. 3).

Other workers have shown that the lymph node proliferative response to SRBC is T-cell dependent in that it does not occur in T-cell-deprived mice (18). However, it has also been suggested that the adjuvant effect of *C. parvum* on the SRBC response might involve a T-cell-bypass mechanism (8). To test whether such a bypass was operating in the present experiments, they were repeated with T-cell-deprived mice (see histograms, Fig. 3). Such mice were incapable of responding to SRBC whether they had been *C. parvum* pretreated or not, which showed the response to be entirely T-cell dependent following *C. parvum* treatment.

The Effect of C. parvum Pretreatment on Loss of Sensitized Cells from Lymph Nodes

Since antigen sensitization was apparently unimpaired in *C. parvum*-treated animals, it was next considered that the sensitized cells may be less well able to leave the node and get into circulation. Lymphocyte trapping has been demonstrated

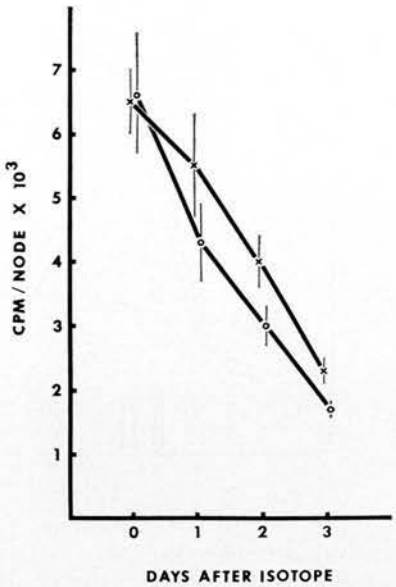

FIG. 4. The effect of *C. parvum* pretreatment on the loss of SRBC-sensitized cells from the popliteal lymph node. Cells dividing in response to SRBC were labeled as follows: 10^8 SRBC were injected into both hind footpads and 20 μCi tritiated thymidine was injected iv 2, 3, and 4 days later. Popliteal lymph nodes were removed and assayed for residual radioactivity 30 min, 1, 2, and 3 days after final isotope injection. Mice received either *C. parvum* (700 μg) iv 7 days prior to SRBC injection (×–×) or no pretreatment (○–○). Vertical bars represent ± standard error (n=5).

to occur following stimulation with various adjuvants, and indeed *C. parvum* is unusual in the marked degree of trapping it causes (6). To follow the fate of cells dividing in response to a footpad injection of SRBC, 10^8 SRBC were injected into both hind footpads of either normal mice, or mice that had received iv *C. parvum* 7 days previously. Two, 3, and 4 days after SRBC, 20 μCi iv of tritiated thymidine was injected into all animals. Lymph nodes were then assayed for residual radioactivity 30 min, 1, 2, or 3 days after the final injection of isotope (Fig. 4). It has been shown that the node DNA synthetic response to SRBC is identical in normal and *C. parvum*-treated mice over the labeling time period (Fig. 3). Presumably some cells are leaving the node over this period and therefore any retention by the node following *C. parvum* treatment would be expected to result in a higher total amount of label in the first instance. However, 30 min after the final isotope injection, the degree of labeling in control and treated nodes was identical, indicating any such cell loss to have been proceeding at the same rate. Moreover, the subsequent rate of loss of label from both groups was the same (Fig. 4).

The Effect of Splenectomy on C. parvum-mediated Inhibition of DTH

The accumulated data indicating that the lymph node was unaffected by iv *C. parvum* and therefore not instrumental in the inhibition of DTH led to the consideration of the role of the spleen. Intravenous *C. parvum* causes marked splenomegaly, due largely to macrophage proliferation (4), and at 7 days spleen weight had increased three- to fourfold. Surgical splenectomy was performed 3 days prior

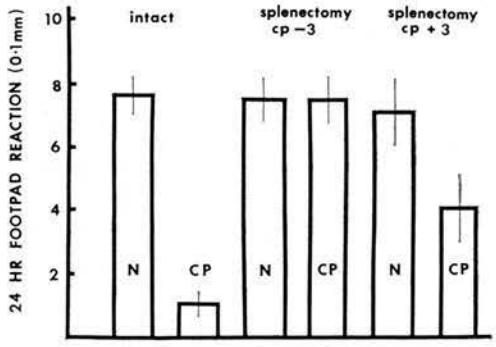

FIG. 5. The effect of splenectomy on DTH elicited by footpad injection of 10^8 SRBC 4 days after sensitization with 10^8 SRBC sc into the neck in either normal mice (N) or in mice that had received *C. parvum* (700 μg) 7 days prior to sensitization (CP). Splenectomy was performed either 3 days before (cp−3) or 3 days after (cp+3) *C. parvum*. Vertical bars represent ± standard error (n=5).

to, or 3 days after, iv *C. parvum* injection. Non-*C. parvum*-injected mice were similarly splenectomized, and the requisite intact controls were included. Seven days after *C. parvum,* 10^8 SRBC were injected sc into the neck, and DTH reactions were elicited in the footpad 4 days later (Fig. 5). *C. parvum* pretreatment caused the usual profound depression of DTH in the intact animal. However, in animals that were splenectomized 3 days prior to *C. parvum* injection, the DTH depression was completely abolished. Splenectomy alone did not affect the DTH level. Splenectomy 3 days after *C. parvum* treatment resulted in an incomplete restoration of DTH depression being significant only at the 10% level. Again, splenectomy itself caused no significant change in DTH levels of untreated animals.

The Effect of Serum from C. parvum-Stimulated Animals

There was the possibility that it might be a humoral product of the *C. parvum*-stimulated spleen rather than the spleen itself which was mediating DTH depression. In mind was antibody to *C. parvum,* since splenectomy is known virtually to abolish antibody production to iv injected particulate antigens. To test this, serum was prepared from mice which had received *C. parvum* iv 10 days previously. (High titers of agglutinating antibody to *C. parvum* at this time had been previously demonstrated—unpublished results, M. T. S.). Five-tenths milliliter of undiluted serum was injected iv into normal mice, followed immediately by a sensitizing dose of SRBC into the neck; DTH was elicited in the footpad 4 days later. Control mice received normal serum. Neither normal nor *C. parvum* serum caused any detectable change in DTH reactivity compared with untreated mice. Further experiments in which the immune serum was mixed with *C. parvum* prior to injection (i.e., to form antigen–antibody complexes) were similarly without effect.

The Effect of C. parvum on Cell Trapping by the Spleen

The mandatory role of the spleen in DTH depression, combined with the apparently normal sensitization of SRBC reactive cells and their subsequent loss from the regional lymph node, were suggestive of trapping of such cells in the *C. parvum*-stimulated spleen. Local administration of *C. parvum* causes increased

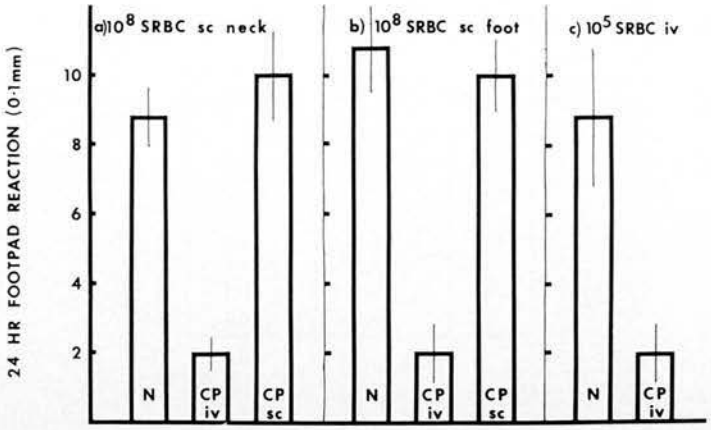

FIG. 6. Comparisons of iv and sc *C. parvum* pretreatment on DTH to subsequently injected SRBC. (a) DTH elicited by footpad injection of 10^8 SRBC 4 days sensitization with 10^8 SRBC sc into the neck in normal mice (N), mice that received *C. parvum* (700 μg) iv 7 days prior to sensitization (CP iv), and mice that received *C. parvum* (700 μg) sc into the neck 7 days prior to sensitization (CP sc). (b) DTH elicited as above in the right hind footpad 4 days after sensitization with 10^8 SRBC sc into the left hind footpad in normal mice (N) or mice that had received *C. parvum* (350 μg) sc into the left hind foot 7 days prior to sensitization (CP sc) or mice that received *C. parvum* (350 μg) iv 7 days prior to sensitization (CP iv). (c) Delayed-type hypersensitivity elicited by footpad injection of 10^8 SRBC 4 days after sensitization with 10^5 SRBC iv in either normal mice (N) or mice that had received *C. parvum* (700 μg) iv 7 days prior to sensitization. (a–c) Vertical bars represent $\pm$ standard error (n=5).

lymphocyte trapping in the draining nodes, as demonstrated by increased uptake of subsequently injected labeled lymphocytes (19), and it was fully expected that this would also be true of the spleen following iv *C. parvum*. To confirm this, labeled SRBC reactive cells were prepared as follows: mice were injected with 10^8 SRBC sc into both hind footpads, and 20 μCi of tritiated thymidine was injected iv 3 and 4 days later. One hour after the final isotope injection, popliteal lymph nodes were removed and cell suspensions were prepared by gentle filtration through a fine stainless-steel sieve. The suspensions were washed and filtered through cotton gauze, and 5×10^7 cells were injected iv into either normal mice or mice which had received iv *C. parvum* 7 days previously. Immediately after cell transfer and 4 and 12 hr later, the recipients received cold thymidine (1000 times the isotope dose) sc into the neck. Spleens were removed for assay of their isotope content at 24 hr. The proportion of the injected cells taken up by the normal and *C. parvum*-stimulated spleens may be compared by considering the total amount of isotope acquired per spleen. The counts per minute per spleen were 2879$\pm$248 (SE) and 5361$\pm$427 for the normal and *C. parvum*-stimulated spleens respectively, which reflects an increased uptake following iv *C. parvum*.

The Effect of Direct Stimulation of Lymph Nodes by C. parvum

The fact that splenectomy prior to *C. parvum* injection completely restored DTH responsiveness suggested that the lymphoid elements remaining after splenectomy were incapable of mediating DTH depression. However, iv *C. parvum* causes relatively little stimulation of the peripheral lymph nodes, since the bulk of the

TABLE 1

STIMULATION OF SPLEEN AND POPLITEAL LYMPH NODE 7 DAYS AFTER IV OR SC *C. Parvum*, RESPECTIVELY

	Organ mass[a]	DNA/organ[a]
Spleen iv CP[b]	4.5	4.4
Lymph node sc CP[c]	5.8	4.6

[a] Experimental/control.
[b] 700 μg *C. parvum* iv.
[c] 700 μg *C. parvum* sc in thigh immediately above popliteal node.

inoculum is taken up by the liver and spleen. It was therefore decided to stimulate the lymph nodes directly with *C. parvum* prior to their processing of injected antigen. In the first series of experiments *C. parvum* was injected either iv or sc into the neck, and 7 days later 10^8 SRBC were injected sc into the neck. Delayed-type-hypersensitivity was elicited in a hind footpad 4 days later. The usual depression of DTH resulted from the iv *C. parvum* injection. However, animals which had received *C. parvum* sc were fully reactive (Fig. 6a).

Injection of material sc into the neck region may stimulate a variety of regional lymph nodes, depending on the exact location of the injections. It was considered that should the injection sites of *C. parvum* and SRBC not be coincident, then antigen may have "escaped" through lymph nodes either unstimulated or not sufficiently stimulated with *C. parvum*. To eliminate this, a second series of experiments was undertaken to ensure passage of antigen through a maximally stimulated node. *C. parvum* was injected iv, as before, or sc into the right thigh immediately above the popliteal node, and 7 days later 10^8 SRBC were injected into the right hind footpad. DTH was elicited in the left hind footpad 4 days later. Intravenous *C. parvum* again depressed DTH reactivity, and again mice which had received the adjuvant sc were fully reactive (Fig. 6b). To confirm and also to assess the degree of stimulation of the popliteal lymph nodes after injection of *C. parvum* into the thigh, groups of mice were assayed for increase in popliteal lymph node mass and DNA synthesis after 7 days. Table 1 compares these results with those for the spleen 7 days after iv *C. parvum* and shows them to be similar in magnitude.

A control omitted from the above experiments is the level of DTH reactivity resulting from antigen processed directly by the *C. parvum*-stimulated spleen—i.e., after iv SRBC sensitization. The reason is that for the antigen doses used only sc sensitization resulted in DTH development (see Figs. 1a and 1b). Very low doses of iv SRBC were, however, found to cause DTH, and mice were sensitized with 10^5 SRBC iv 7 days after iv *C. parvum*. Delayed-type hypersensitivity was elicited 4 days later, and the resulting depression was similar to that following sc sensitization (Fig. 6c).

DISCUSSION

The presented observations that iv *C. parvum* pretreatment reduces DTH reactivity contribute to the accumulating evidence that, despite its profound potentiating

effect on B cells (3, 4) and its marked anti-tumor effect (5–7), T-cell responses are inhibited by iv *C. parvum*. Thus far, PHA, MLR, GVH, (12) contact sensitivity (14), and homograft reactivity (Castro, quoted in Ref. 8) are reported to be depressed by this adjuvant. Cell-mediated immunity and humoral immunity are considered to coexist in balance (20), but the combination of iv *C. parvum* followed by sc SRBC shows DTH reactivity to be markedly depressed without a concomitant increase in circulating antibody levels. Increase in antibody levels did occur when the conventional adjuvant regimen of iv *C. parvum* prior to iv antigen was used; however, Arthus reactivity was not modified. In similar experiments showing Freund's complete adjuvant to depress DTH reactivity to picryl chloride, both immediate and delayed hypersensitivities were reduced (14).

Sensitization at the level of the lymph node draining the site of antigen injection in animals pretreated with iv *C. parvum* was not impaired as assessed by the degree of antigen-induced DNA synthesis. That iv *C. parvum* had influenced the popliteal node was evident from the slight augmentation of the node DNA response. The kinetics of this modified response are strikingly similar to those described for the adjuvant activity of *C. parvum* on antibody-forming cells as measured by both rosetting (3) and plaquing (4) techniques; i.e., the early time course of the augmented response is similar to the control, but it reaches a higher peak and is more sustained. The DNA synthetic response to SRBC described here has been shown to be entirely T-cell dependent in both normal and *C. parvum*-treated animals, and there is no evidence for any *C. parvum*-mediated T-cell bypass mechanism as has been reported to occur with the 7S spleen plaque-forming cell response to SRBC (8).

Splenectomy 3 days prior to *C. parvum* injection abolished the subsequent DTH depression, not only showing that the depressive effect is mediated by the *C. parvum*-stimulated spleen, but also indicating that the remaining lymphoid tissue is functionally incapable of replacing the spleen in this respect. This was borne out by the subsequent finding that direct stimulation of lymph nodes with *C. parvum* prior to their processing antigen did not result in DTH depression as discussed below. Another interesting point is that the liver, which is highly stimulated following iv *C. parvum* (1), also apparently plays no part in the depression of DTH. That the effect is mediated by the cells in the spleen rather than any humoral product of the spleen is suggested by the failure to depress DTH by large amounts of serum from *C. parvum*-stimulated animals. Splenectomy 4 days after *C. parvum* administration significantly restored DTH responsiveness but markedly less so than prior splenectomy. This residual depression coupled with demonstrated ineffectiveness of lymph nodes may indicate a migration of the relevant cell type from the spleen to other sites.

The finding that there is no T-cell impairment at the lymph node level following iv *C. parvum,* but that it is the spleen that is responsible for DTH depression, is in full accord with previous *in vitro* findings concerning T-cell inhibition by *C. parvum*. Spleen cells from mice pretreated with iv *C. parvum* were unresponsive to PHA, whereas lymph node performance was unimpaired (12). This inhibition of PHA responsiveness *in vitro* has been shown to be mediated by the influence of *C. parvum*-activated macrophages on the potentially reactive T cells (13). The interaction apparently required contact between the two cell types, which again accords with the present finding that it is the spleen cells themselves, rather than any humoral

product, that are instrumental in DTH depression. These and further similarities which will emerge later in the discussion are consistent with the idea that such a *C. parvum*-activated macrophage inhibition of T-cell activity is operative both *in vitro* and *in vivo*.

Impairment of the ability of T cells to divide in response to PHA *in vitro* suggests that there may be a similar impairment of T cells dividing in response to antigen *in vivo*. This is not true, however, in the present system where normal sensitization is demonstrated to occur in the peripheral nodes. Subsequent loss of these cells from the nodes is also unimpaired, and the demonstrated increased cell trapping by the *C. parvum*-stimulated spleen would readily account for their arrival in the spleen. Adjuvants have recently been shown to cause lymphocyte trapping which lasts over several weeks at a high sustained level and *C. parvum* has been commented upon for the marked magnitude of trapping it causes (19). The increased splenic trapping described here may merely reflect the increased filtering capacity of the enlarged spleen; however, it is interesting to note that the same cell type, the activated macrophage, that is deemed to be instrumental in the reported immunosuppressive and immunostimulatory effects of *C. parvum* to date (4, 8, 13) is now also considered to mediate the lymphocyte-trapping phenomenon (21). The fate of the sensitized T cells on reaching the spleen may be conjectured—they may be prevented from undergoing further division or they, and any progeny, may merely be prevented from leaving the spleen. Whatever, the end result is presently considered to be reduced distribution of the sensitized cells throughout peripheral lymphoid tissue—i.e., those nodes serving the area where the DTH reaction is elicited. Such a restricted lymphocyte movement following iv *C. parvum* has been described recently where there was a failure to recruit lymphocytes by lymph nodes undergoing antigen stimulation in *C. parvum*-treated mice (22); however, no increased splenic uptake was demonstrable in these experiments.

The inability of the lymph nodes to substitute for the spleen in DTH depression is interesting, especially when the node itself was stimulated directly with *C. parvum* prior to antigen processing. The degree of stimulation was similar to that of the spleen following iv *C. parvum*, as evidenced by both increase in mass and DNA synthesis. Preliminary histological examination has also shown macrophage accumulation in such nodes. Local injection of *C. parvum* is known to result in marked lymphocyte trapping in the draining lymph nodes (19, 21), and yet no DTH depression occurs. This suggests that the mere retention of sensitized cells by the spleen may, of itself, be insufficient to cause DTH depression, and that the phenomenon is more dependent on the subsequent fate of such cells in the spleen. The discrepancy between the performance of stimulated spleen and lymph nodes directly parallels that noted for the PHA response *in vitro*, where the responsiveness of iv *C. parvum*-stimulated spleen cells was abolished, but cells from popliteal lymph nodes stimulated directly with *C. parvum* responded normally to optimal concentrations of PHA (12). This further implies that the same *C. parvum*-activated macrophage–T-cell interaction (13) is instrumental in DTH depression *in vivo*. Recently, Gillette and Boone described the splenic PHA response of tumor-bearing animals to be depressed and were intrigued to find that lymph node cells were not similarly affected (23). RES activity is known to be stimulated in animals bearing a variety of transplantable tumors (24) and, furthermore macrophages activated by means other than *C. parvum* have been demonstrated to be

capable of depressing T-cell activity *in vitro* (13). This then may be additional evidence for the discrepancy between the performance of lymph node and spleen with regard to T-cell inhibition. Short of the effect being attributable to merely quantitative differences between the spleen and lymph node macrophage populations then, the data may indicate either different kinds of macrophages or possibly different anatomical distributions of macrophages in these organs. The correlation between the *in vitro* and *in vivo* data certainly strongly implicates the macrophage, but it was hoped to demonstrate their *in vivo* role less equivocally by using crystalline silica, an agent selectively toxic for macrophages. These experiments were, however, thwarted by the finding that the silica itself depressed DTH reactivity.

That *C. parvum* is considerably less effective in depressing DTH reactivity when injected simultaneously or after antigen is in keeping with the finite time required for *C. parvum* macrophage activation to occur (8) and the idea that sensitized cells would be free to distribute themselves normally before the onset of trapping. The finding is also relevant to the use of *C. parvum* in tumor therapy, since treatment of an established tumor means that sensitization with tumor antigens must in large part precede *C. parvum* and depression of cell-mediated immunity may be thereby circumvented. An ongoing tumor may, however, serve as a source of continuous antigen stimulation, and splenectomy as an adjunct to iv *C. parvum* therapy deserves consideration. In addition, sc administration of *C. parvum* did not cause depression of cell-mediated immunity, and this route of injection has been shown to be effective against tumors, at least when combined with chemotherapy (25, 26) or injected either directly into the tumor site or at a distant site prepared with irradiated tumors cells (Scott, in preparation).

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Enhanced Resistance of Mice to Infection with Bacteria following Pre-treatment with *Corynebacterium parvum*

KILLED suspensions of *Corynebacterium parvum* have been shown to stimulate the lymphoreticular system[1] and to possess adjuvant activity[2,3]. Pre-treatment of animals with *C. parvum* increases their resistance to tumour cell challenge[4,5] and to protozoal infection[6]. In view of these properties it seemed probable that *C. parvum* might induce protection against bacterial challenge. We therefore examined the effect of *C. parvum* treatment on the course of three bacterial infections in W-Swiss mice. The strain of *C. parvum* used was supplied by Professor M. Raynaud of the Pasteur Institute and has been given the Wellcome Culture Collection number CN 6134.

Table 1 shows the results of an experiment in which mice received a single intravenous injection of killed *C. parvum* before an intraperitoneal challenge of living *Brucella abortus* (strain 1914). Considerable protection was afforded to animals challenged 1 day to 4 weeks after *C. parvum* treatment. Maximal effect was observed after 4 days.

The effect of *C. parvum* treatment on the susceptibility of mice to *Bordetella pertussis* infection is shown in Table 2. Mice which had received a single injection of *C. parvum* (intravenous or intraperitoneal) were challenged intracranially with living, mouse-virulent *Bord. pertussis* (strain 18/323). The LD_{50} of this strain by this route was less than 100 organisms. Pre-treatment of animals with *C. parvum* resulted in marked resistance to a *Bord. pertussis* challenge of approximately 400 LD_{50}s. In contrast to the protection shown against *Br. abortus*, resistance to *Bord. pertussis* reached a maximum 2 to 3 weeks after *C. parvum* injection. The degree of protection was similar when *C. parvum* was injected intravenously or intraperitoneally.

When *C. parvum*-treated mice were challenged intravenously with *Staphylococcus aureus* (Wellcome Culture Collection No. CN 57) no consistent protective effect was observed. Protection was, however, apparent when an intraperitoneal *C. parvum* injection preceded an intraperitoneal staphylococcal challenge (Table 3).

Experiments are in progress to determine the mechanisms by which *C. parvum* produces its protective effects in the

Table 1 Effect of *C. parvum* * on the Susceptibility of Mice to *Br. abortus*

i.p. *Brucella* challenge No. organisms/ mouse	Interval between *C. parvum* pre-treatment and challenge and No. survivors/ten mice								
	−18 h †	4 h	1 d	4 d	1 w	2 w	3 w	4 w	Untreated controls
1.1×10^9	ND ‡	0	2	4	1	2	2	0	ND
5.6×10^8	0	0	8	7	6	4	3	4/9	1
2.8×10^8	0	0	9	10	9	10	5	5/9	1
1.4×10^8	1	5	10	10	10	10	10	9	6
7.0×10^7	10	9	10	10	10	10	9	10	9
$LD_{50} \times 10^8$	0.9	1.3	7.8	9.3	6.2	6.1	4.0	3.6	1.6
lfiducial (imits)	(0.6–1.3)	(0.9–1.8)	(5.6–13.0)	(6.6–20.6)	(4.6–8.9)	(4.2–8.8)	(2.6–6.4)	(2.5–5.4)	(1.2–2.4)

* 1.4 mg dry weight, intravenously/mouse.
† This group received *C. parvum* 18 h after challenge. Deaths were recorded for 14 days after challenge.
‡ Not done.

systems described. Both adjuvant action and reticulo-endothelial stimulation may be important. Fauve and Hevin[7] have recently shown that spleen and liver macrophages taken from *C. parvum* treated animals possess greater bactericidal powers than control macrophages and this may be the mechanism by which brucella organisms are destroyed in the *C. parvum* treated animal. The failure of *C. parvum* to protect against an intravenous staphylococcal injection may be due to the predilection of these organisms for the kidney[8], an organ which is not believed to be affected by lympho-reticular stimulation.

Table 2 Effect of *C. parvum* * on the Susceptibility of Mice to *Bord. pertussis* †

Route of injection of *C. parvum*	Days between *C. parvum* pre-treatment and challenge and No. survivors/No. challenged						
	−3 ‡	0	4	7	14	21	28
i.v.	0/10 §	0/9	0/5	0/7	5/9	7/10	7/9
i.p.	0/10	0/10	0/10	3/10	5/10	3/9	6/10

* 1.4 mg dry weight/mouse.
† 3.7×10^4 viable organisms/mouse.
‡ This group received *C. parvum* 3 days after challenge.
§ Deaths were recorded for 14 days. Deaths up to 3 days after challenge were discounted.

Table 3 Effect of *C. parvum* * on the Susceptibility of Mice to Intraperitoneal Challenge with *Staph. aureus* †

Days between *C. parvum* pre-treatment and challenge	Route of injection of *C. parvum*	Days after challenge with staphylococci 0	1	2	4	14	21
0	i.p.	10 ‡	0	0	0	0	0
	i.v.	8	0	0	0	0	0
4	i.p.	10	10	10	9	5	4
	i.v.	10	3	0	0	0	0
7	i.p.	10	10	8	8	5	5
	i.v.	9	4	1	0	0	0
14	i.p.	10	10	9	9	6	5
	i.v.	8	6	5	5	1	1
21	i.p.	9	6	3	3	2	1
	i.v.	9	9	3	2	0	0
28	i.p.	9	7	4	3	2	2
	i.v.	9	6	0	0	0	0
Untreated controls		7	4	0	0	0	0

* 1.4 mg dry weight/mouse.
† 7.1×10^7 viable organisms/mouse.
‡ Figures in the body of the table are survivors at days after challenge.

Some other mechanism must, however, be acting to protect *C. parvum* treated mice from lethal intracerebral *Bord. pertussis* challenge and it may be significant that *C. parvum* like *Bord. pertussis* (and to some extent brucella)[9] can sensitize mice to histamine. Table 4 shows the results of an experiment in which mice were challenged with histamine phosphate at vary-

Table 4 Effect of *C. parvum* * on the Susceptibility of Mice to Histamine

Treatment	Dose of histamine base/mouse (mg)	Interval between treatment and challenge and No. survivors/twenty mice 1 h	4 h	12 h	24 h	4 d	7 d	14 d	21 d	28 d
Saline	5	20	20	18	20	20	19	20	19	19
C. parvum	1	18	13	15	15	3	2	4	19	20

* 1.4 mg dry weight intravenously/mouse.

ing times after *C. parvum* treatment. Maximal susceptibility to histamine was demonstrable 4 to 7 days after *C. parvum*. Most deaths occurred within 2 h of histamine challenge.

The separate identity of the histamine sensitizing factor and the intracerebral protective antigen of *Bord. pertussis* have been a matter of controversy[9]. Although *C. parvum* and *Bord. pertussis* do not cross react antigenically as judged by bacterial agglutination, the fact that both protect against the same challenge in the same system suggests the existence of some unidentified relationship.

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Effect of *Corynebacterium parvum* Treatment on the Growth of *Salmonella enteritidis* in Mice

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The growth of *Salmonella enteritidis* in mice pretreated with 700 μg of killed *Corynebacterium parvum* was less than that seen in normal CD-1 mice. In treated mice, there was an early increased inactivation of the blood, liver, and spleen bacterial populations, followed by a prolonged period of slow but continuous bacterial growth. The treated mice failed to develop significant delayed hypersensitivity and did not show the characteristic antibacterial immune response seen in untreated infected animals. Eventually sufficient resistance did develop in most of the treated animals to protect them against the lethal effects of the challenge infection. The peak *C. parvum* effect was seen when *S. enteritidis* was injected 7 to 14 days later. Injection of *C. parvum* 24 h after the bacterial challenge actually potentiated the *Salmonella* infection. There was no evidence of an increased specific humoral response by the *C. parvum*-treated mice, suggesting that the slower growth of the *S. enteritidis* was due to the continued enhanced killing of the bacterial population by the nonspecifically stimulated cells of the reticuloendothelial system, rather than to any specific augmentation of the host immune response.

Intravenous injection of killed *Corynebacterium parvum* into normal mice (13) results in an intense stimulation of the reticuloendothelial system (RES). There is an associated increase in resistance to some acute bacterial (1) and protozoan (18) infections and even to some tumor transplants (21). Part of this augmentation of the host's response after the introduction of *C. parvum* into the tissues may be explained by its adjuvant action (3, 14) and, since both humoral and cellular defenses are involved in acquired resistance to many facultative intracellular parasites (8, 16), any stimulus to the production of specific humoral factors could increase host resistance by promoting phagocytosis and the early inactivation of the challenge population. There is good evidence, however, that the elimination of intracellular microbial parasites often depends upon a cell-mediated immune response with a resulting state of delayed-type hypersensitivity (DTH) being developed against one or more microbial antigens (16, 17). There is an apparent contradiction between the overall protection afforded by *C. parvum* given before infection (1) and the depressive effect of this agent on T-cell reactivity (2, 19, 20). This paradox would be resolved if host resistance in the *C. parvum*-treated animal was found to depend upon nonspecific activation of the RES (12, 13).

The present study examines the effect of intravenously injected *C. parvum* on the development of DTH and acquired resistance to *Salmonella enteritidis*. DTH to the *Salmonella* test antigen was found to be severely impaired in the *C. parvum*-treated mice, which were, nonetheless, resistant to the challenge, showed increased rates of blood clearance, and had the capacity to inactivate an enlarged proportion of the challenge inoculum. These are all features that commonly result from stimulation of the RES. No doubt they are responsible for the survival advantage conferred on the mice by *C. parvum* treatment. However, the simultaneous depression of cell-mediated immunity in these animals was probably responsible for the slow eradication of organisms from the liver and spleen and so contributed to the protracted salmonella infections observed in some of the *C. parvum*-treated mice.

MATERIALS AND METHODS

Animals. Specific pathogen-free CD-1 mice (Charles River Farms, Wilmington, Mass.) were maintained, 10 to a cage, on sterile bedding and kept under Isocaps (Carworth Lab Cages, N.Y.) to prevent cross-infection (9). They were fed sterile pellets and water ad lib. Female mice weighing 18 to 24 g were used throughout.

Organisms. *S. enteritidis* NCTC 5694 and a strep-

tomycin-resistant variant (SM^R) were maintained under conditions described previously (5). Both strains were virulent for CD-1 mice (intravenous mean lethal dose (LD_{50}) = 5 to 10×10^3). The method for determining the LD_{50} values has been described elsewhere (9). Inocula were grown overnight at 37 C in tryptose soy broth (Difco), and then subcultured into 5 ml of fresh broth and incubated for 5 h at 37 C. The culture was harvested during the late logarithmic phase and diluted suitably in sterile saline immediately before injection into the mice. The number of viable organisms in the inoculum was checked by plating samples onto tryptose soy agar (TSA) and incubating overnight at 37 C.

Killed *C. parvum* suspension was kindly provided by C. Adlam, Wellcome Laboratories, Beckenham, Kent, U.K. The suspension contained 7 mg (dry weight) of cells per ml. Mice were usually injected intravenously with 0.1 ml of suspension 7 to 10 days prior to challenge. Heat-killed *Mycobacterium tuberculosis* $H_{37}R_v$ (11) was homogenized in sterile saline to give a suspension containing 7 mg (dry weight) of cells per ml, and mice were injected intravenously with 0.1 ml of suspension and tested 7 days later. Heat-killed *S. enteritidis* (60 C for 60 min) was homogenized in sterile saline (7 mg/ml), and 0.1 ml was injected intravenously into CD-1 mice. The mice initially showed some endotoxic reaction, but only two of the 50 mice died.

Enumeration of bacteria in vivo. The livers and spleens were removed aseptically from groups of five randomly selected mice and homogenized separately in cold sterile saline. The homogenates were diluted suitably and plated on TSA (6). Heart blood (0.1 ml) was plated onto TSA. In challenge experiments, the homogenates were plated on TSA, with or without 20 μg of streptomycin per ml of agar. Separate counts of the drug-resistant challenge and drug-sensitive residual vaccinating populations were recorded.

DTH determinations. Mice were injected in one hind footpad with 2.0 μg of protein test antigen diluted in 0.03 ml of sterile saline. The antigen was obtained from a 72-h culture filtrate of *S. enteritidis* as described earlier (10). The foot swelling, with reference to the contralateral foot, was measured at 3 and 24 h with dial gauge calipers (Schnelltäster, Kroplin). An increase of 1.8 U or more (0.18 mm) was significant at the 1% level.

Serology. Mice were bled by heart puncture, and the serum was separated and heated at 56 C for 30 min before storage at −20 C. Hemagglutinin titers were measured by using alkali-treated *S. enteritidis* lipopolysaccharide (Difco) absorbed onto washed sheep red blood cells (4). The reciprocals of the serum titers were expressed as $\log_2$ values (± standard error).

RESULTS

Growth of S. enteritidis in C. parvum-treated mice. Normal mice were injected intravenously with 700 μg of *C. parvum* in 0.1 ml of saline or with 0.1 ml of saline alone, and 7 days later the two groups were infected intravenously with about 10^4 viable *S. enteritidis* 5694. The challenge organism was rapidly cleared from the blood of the *C. parvum* mice and taken up in the liver and spleen in approximately equal numbers (Fig. 1). Both the liver and spleen bacterial counts declined substantially over the first 150 min of the challenge period so that by this time the survivors represented only 1% of the original inoculum. This early, extensive inactivation of the challenge infection contrasts with the incomplete blood clearance and slower rate in decline shown by the liver and spleen populations in the control animals (Fig. 1). As a result, up to 20% of the inoculum survived the initial inactivation period, and this 10-fold difference in the size of the 24-h challenge populations in the two groups of mice was sufficient to account for the higher survival of the *C. parvum*-treated animals (Fig. 2). Thus, it can be said that *C. parvum* treatment, by markedly stimulating both blood clearance and bacterial inactivation (clearly the effect of a nonspecific increase in phagocytic function),

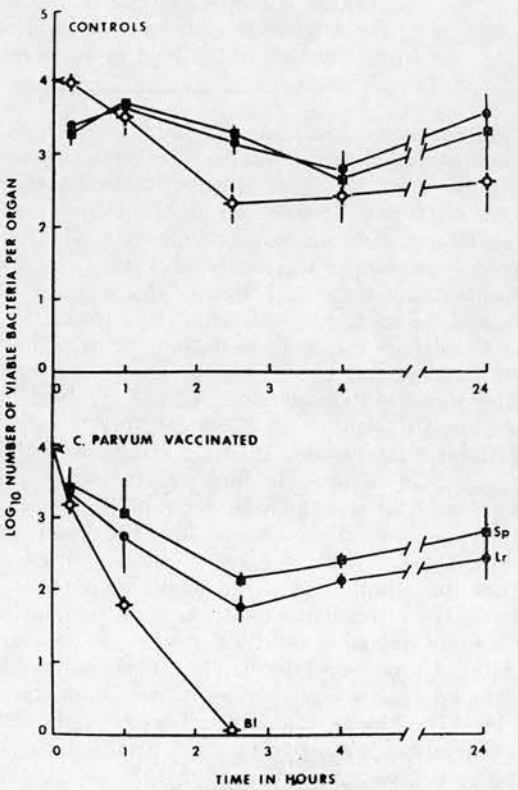

FIG. 1. *Viable counts of S. enteritidis in the liver (Lr), spleen (Sp) and blood (Bl) of C. parvum-treated mice (bottom) or in normal controls (top) over the first 24 h of the infection.*

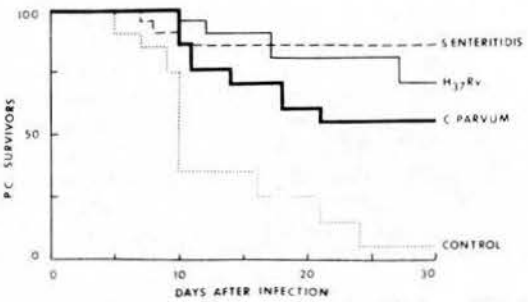

FIG. 2. *Survival of mice treated with heat-killed bacterial suspensions 7 days prior to challenge with 10^4 viable S. enteritidis.*

conferred a clear-cut survival advantage on treated animals. However, once this initial inactivation was complete, the surviving *S. enteritidis* in the *C. parvum*-treated mice began to multiply in the liver and spleen, though at a slower rate than in untreated controls (Fig. 3).

The typical immune response developed by untreated mice was characterized by a sharp change in growth rate of the *Salmonellae* about day 6, and this was associated with the development of high levels of DTH with little evidence of Arthus reactivity (Fig. 3). This has been taken as evidence of a cell-mediated immune response against the infecting organism (5, 7). On the other hand, the *C. parvum*-treated mice developed considerable Arthus (3 h) footpad sensitivity after the *Salmonella* challenge, but most of this foot swelling had disappeared by 24 h (Fig. 3), suggesting that the development of DTH had been impaired by the *C. parvum* treatment. This apparent absence of DTH correlated with the inability of the treated animals to eradicate the challenge organisms from the tissues.

Reinfection of the *S. enteritidis*-infected mice on day 12 with 10^4 viable *S. enteritidis* SM^r was associated with an immediate inactivation of the drug-resistant population in both liver and spleen (Fig. 3). However, the rate of decline of viable SM^r organisms in the *C. parvum*-treated mice was considerably slower than in the corresponding controls; 7 days were required to inactivate 99% of the challenge inoculum in the treated group compared with 2 days for a similar kill in the controls. Much reduced levels of DTH were also observed in the *C. parvum*-treated mice after reinfection. This again points to an impairment of cell-mediated immunity in these animals.

A curious feature of the *Salmonella* growth curves observed in all of the *C. parvum*-treated mice was a reversal of the initial distribution of the challenge population between the liver and spleen (Fig. 1, 3). The relative proportions of both the liver and spleen bacterial populations frequently remained inverted until the infection reached near lethal proportions. The reason for this shift and its significance concerning the evolution of the infection in the treated animal is still unknown.

The early inactivation of an intravenous inoculum of *S. enteritidis* and its subsequently slower rate of growth in the *C. parvum*-treated mice could theoretically have been due to an enhanced production of specific opsonins against the somatic *Salmonella* antigens. However, passive hemagglutination tests using purified *S. enteritidis* lipopolysaccharide against sera taken from *C. parvum*-treated mice failed to reveal detectable levels of such antibodies (Table 1). Even when these mice were challenged with *S. enteritidis*, the hemagglutinin titers found in sera harvested on day 12 of the infection were still significantly lower ($P < 0.01$) than they were for the *Salmonella*-infected controls. This depressed humoral response by the *C. parvum*-treated animal was ascribed to the lowered bacterial population and, thus, to a smaller antigenic stimulus. For that matter, the reduced growth by *S. enteritidis* could also be thought to account for the apparent absence of DTH in treated mice. If this were so, it should be possible to induce a normal cell-mediated

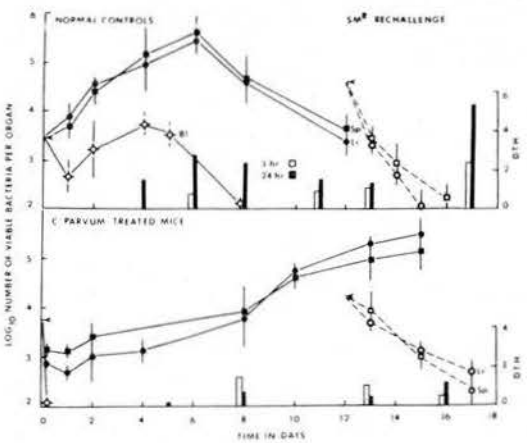

FIG. 3. *Growth of S. enteritidis in normal (top) or C. parvum-treated mice (bottom) after intravenous challenge; Sp, spleen; Lr, liver; Bl, blood. Broken lines represent a streptomycin-resistant rechallenge population injected on day 12. Open histograms represent the average 3-h foot swelling (average of five determinations), and the solid bars represent the 24-h increases in average foot thickness following injection of 2 μg of Salmonella test antigen.*

TABLE 1. *Hemagglutination titers for mouse sera after killed C. parvum or S. enteritidis pretreatment followed 7 days later by infection with living S. enteritidis*

Pretreatment	Days after infection		
	1	6	12
C. parvum	<2.0[a]	<2.0[b]	3.1 ± 0.25[b]
S. enteritidis	10.0[a]	8.8 ± 1.10	–[c]
Saline	<2.0[a]	3.5 ± 0.45	5.6 ± 0.77
C. parvum without challenge	<2.0[a]	–[c]	–[c]

[a] Log_2 of the inverse hemagglutinin titer for pooled sera.
[b] Mean (± standard error) for five determinations.
[c] Not tested.

immune response merely by increasing the size of the challenge dose 100-fold. Mice were therefore injected intravenously with *C. parvum* 7 days prior to their challenge with 5×10^5 viable *S. enteritidis* (100 LD_{50}'s). The growth rate of the bacterial challenge of the liver and spleen was still markedly reduced compared with the controls (Fig. 4), but the larger bacterial population induced an antibacterial response as judged by the inhibition of further growth by the organisms in the liver and spleen from day 6 onwards. Despite this evidence for a cell-mediated response to the further growth of the massive challenge infection, the *C. parvum*-treated mice developed barely significant levels of DTH (0.15 ± 0.05 mm).

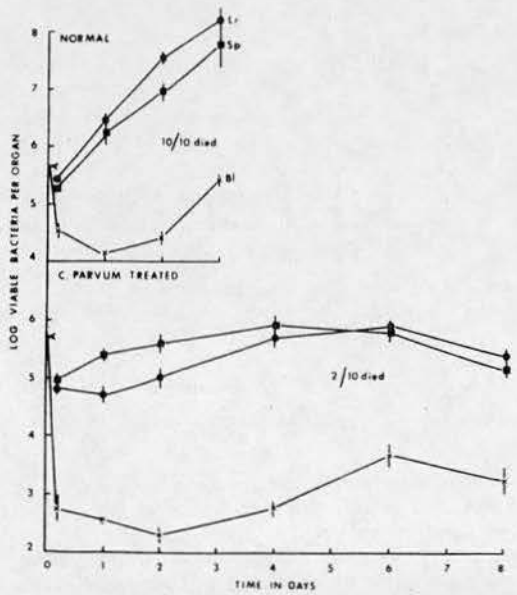

FIG. 4. *Growth of S. enteritidis in C. parvum-treated and control mice (top) after the introduction of 100 LD_{50} dose of virulent organisms by the intravenous route.*

Effect of heat-killed M. tuberculosis or S. enteritidis on anti-Salmonella immunity. There was a possibility that the slowed *Salmonella* growth in the *C. parvum*-treated mice was due to antigenic competition as a result of the massive inoculum of dead corynebacteria. To investigate this possibility, mice were injected with equivalent amounts of two other bacterial suspensions. The first was *M. tuberculosis* $H_{37}R_v$, selected as an unrelated organism unlikely to induce a cross-reacting humoral response, and the second was heat-killed *S. enteritidis*, known to induce high levels of specific antibodies in intravenously vaccinated mice (7). Groups of mice were injected with 1,400, 700, and 350 μg of heat-killed *C. parvum* or *M. tuberculosis* ($H_{37}R_v$) or with either 700 or 350 μg of heat-killed *S. enteritidis* (the 1,400 μg dose proved to be too toxic for the mice). Seven days later, the pretreated mice, together with a group of normal controls, were infected with increasing numbers of viable *S. enteritidis*, and the LD_{50} values for each group were determined (Table 2). Injection of 350 μg of heat-killed *S. enteritidis* or *M. tuberculosis* $H_{37}R_v$ increased the size of the median lethal dose of viable *Salmonellae* approximately five-fold. This should be compared with the 10-fold increase observed in the *C. parvum*-treated animals (Table 2). Increasing the dosage of *C. parvum* or $H_{37}R_v$ to 1,400 μg seemed to have little further protective effect.

Growth studies carried out in *Salmonella*-infected mice pretreated with 700 μg of either heat-killed $H_{37}R_v$ or *S. enteritidis* indicated that both groups of animals had an increased ability to kill the challenge organism in liver and spleen over the first 24 h, but that this was followed by a nearly normal growth rate until peak counts were observed about day 6 (Fig. 5).

TABLE 2. *Effect of pretreatment of mice with suspensions of various killed bacteria 7 days prior to determination of LD_{50} values for intravenous S. enteritidis 5694*

Pretreatment	Median lethal dose[a]			
	1,400 μg[b]	700 μg	350 μg	0 μg
C. parvum	5.2[a]	4.8	4.75	3.65
M. tuberculosis	4.65	4.6	4.2	3.70
S. enteritidis		4.25	3.95	3.40

[a] Log_{10} of the median lethal dose of viable organisms required to kill 50% of the mice within 28 days of challenge (5, 8).
[b] Intravenous *C. parvum* dosage.

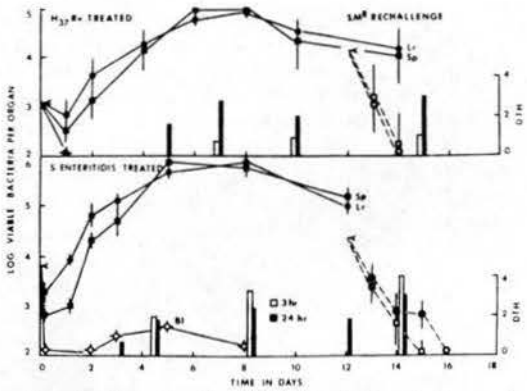

FIG. 5. *Growth of an S. enteritidis challenge in mice treated with 700 μg of heat-killed M. tuberculosis $H_{37}R_v$ (top) or S. enteritidis 5694 (bottom) 7 days previously. Both groups of mice were then reinfected intravenously with 10^4 S. enteritidis SM^R on day 12 (broken lines). Histograms represent footpad reactivity to the Salmonella test antigen.*

Survival curves shown in Fig. 2 indicate that killed $H_{37}R_v$ and *S. enteritidis* both induced substantial protection against the *Salmonella* challenge. The observed differences in percentage of survival in these two groups was probably not significantly different from that obtained with *C. parvum;* however, the growth curves were strikingly different, exhibiting both a normal immune response and high levels of DTH (Fig. 5). Strong Arthus (3 h) reactivity developed in the *S. enteritidis*-treated animals, making the 24-h reactions more difficult to interpret. As expected, the serum antibody levels in these mice were also greatly elevated (Table 1), and blood clearance of the *S. enteritidis* challenge was both rapid and complete (Fig. 5). The response to reinfection with *S. enteritidis* SM^R showed that both the $H_{37}R_v$- and *S. enteritidis*-treated mice were capable of developing high levels of cell-mediated immunity despite the massive antigenic load of killed bacilli introduced into the RES prior to challenge. These results suggest, therefore, that the immune response was impaired only by the *C. parvum*.

Time course of the host response to C. parvum. Groups of mice were injected intravenously with 700 μg of *C. parvum* at various times with respect to the *S. enteritidis* challenge. When *C. parvum* was injected 24 hr after challenge, the infection progressed more rapidly (Fig. 6); when given at the same time, it had little or no effect. When the challenge was delayed for 7 or 14 days after the *C. parvum*, the growth of *S. enteritidis* in both the liver and spleen was depressed. However, if the time interval between treatment and challenge was further increased to 28 days, the growth curves for *S. enteritidis* returned substantially towards normal, and DTH was again a feature of the host response (Fig. 6).

DISCUSSION

In terms of host survival (1), *C. parvum* has a protective effect if given before infection with *S. enteritidis*, but not after (Fig. 6). Because *C. parvum* clearly has a marked stimulatory effect on the RES (13), it is probably this factor that was responsible for the increase in survival shown in Fig. 2, but *C. parvum* has also been reported to act as an immunological adjuvant for a variety of unrelated antigens (3, 14). Thus, the presence of *C. parvum* could have stimulated an accelerated humoral response to the surface antigens of *S. enteritidis*. This possibility seems unlikely, however, in view of our inability to detect hemagglutinins in the serum of *C. parvum*-treated mice at the time of challenge or even quite late in the *Salmonella* infection (Table 1). Substantial antibody titers were detected in mice given 700 μg of heat-killed *S. enteritidis*, but the growth patterns of the challenge *Salmonellae* remained essentially normal (8), whereas those for animals treated

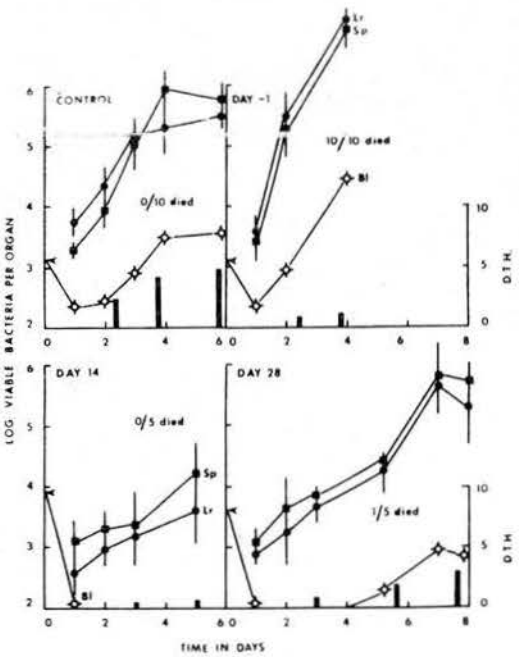

FIG. 6. *Growth of S. enteritidis in mice receiving 700 μg of heat-killed C. parvum one day after the Salmonella challenge (top right) or 14 or 28 days prior to infection with 10^4 viable S. enteritidis (bottom). Histograms represent the DTH reactions.*

with *C. parvum* were not. Whereas minute traces of specific or cross-reacting opsonin may be produced in *C. parvum*-treated mice (thus accounting for the increased blood clearance and inactivation of organisms during the early hours of infection), such factors can hardly explain the continued slower growth of the *Salmonellae* over the next 14 days. On the other hand, the presence of large numbers of nonspecifically activated macrophages in the grossly enlarged livers and spleens of the *C. parvum*-treated animals could limit bacterial growth in vivo for as long as the stimulatory effect of the *C. parvum* persisted. It is known that *C. parvum*-stimulated macrophages do have an increased bactericidal capacity (12). It thus seems reasonable to postulate that the restrained growth of the *Salmonellae* in *C. parvum*-treated mice is due primarily to the nonspecific effects of RES activation. However, there is no question that *C. parvum* treatment also reduced the level of DTH in the *Salmonella*-infected mice. Furthermore, a challenge population of drug-resistant *S. enteritidis* was inactivated more slowly in these mice than in control animals. This suggests that an important component of the immune response is not operating normally in the *C. parvum*-treated mouse. This accords with accumulating evidence that *C. parvum* tends to inhibit T-cell function. The response to phytohemagglutinin, the mixed lymphocyte reaction, the graft-versus-host reaction (19), and the development of contact sensitivity (2) have all been reported to be depressed in mice treated with *C. parvum*.

Regardless of the mechanism involved, the *C. parvum* effect reached a maximum about day 7, but when the challenge and the *C. parvum* were injected simultaneously or even 24 h apart, no obvious change in the *Salmonella* growth curve could be seen (Fig. 6). The time lag before the treated mice displayed a depressed ability to develop DTH presumably represents the time required for activated macrophages to develop in response to the *C. parvum* stimulus (15). This view is consistent with the idea that the activated macrophages in the liver and spleen not only directly restrain the growth of the *Salmonellae* in vivo, but are also responsible for the treated animal's inability to develop an effective antibacterial resistance to the residual challenge infection. However, it is interesting to note that depression of T-cell function by *C. parvum*-activated macrophages has been described to occur in vitro (20). The picture of an overall increased resistance to a lethal challenge infection in the face of an apparently reduced capacity to mount a cellular immune response is also similar to that already described for *C. parvum*-treated mice receiving murine tumor transplants (21). It seems reasonable that the *C. parvum*-activated macrophages within the RES could be the primary effector in both systems.

It is interesting to note that bacterial growth was enhanced when *C. parvum* was given 24 h after infection (Fig. 6). This would seem to rule out the use of this reagent for therapeutic purposes. However, the combination of nonspecific antibacterial activity and a possible specific depression of T-cell-mediated immunity (recently extended to include allograft reactions; 15) would seem to raise the possibility of using *C. parvum* to suppress the rejection mechanism while achieving a desirable increase in the level of nonspecific antimicrobial resistance. Such a situation would be an improvement upon the usual consequence of immunosuppressive drug therapy.

ACKNOWLEDGMENTS

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Protection of Mice Against Viral Infection by *Corynebacterium parvum* and *Bordetella pertussis*

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SUMMARY

Mice could be significantly protected against infection with herpes simplex virus (HSV) by i.p. or i.v. injection of killed *Corynebacterium parvum* 7 days before infection. This protection was seen in inbred strains of mice with a different degree of sensitivity to HSV and after both i.p. and i.v. infection. Resistant mice immunosuppressed by X-irradiation and showing an increased susceptibility to HSV could also be protected by a previous injection of *C. parvum*. Elevated levels of interferon were demonstrated in the serum of mice injected with *C. parvum* 5 to 12 days previously. Four different strains of anaerobic coryneforms were compared and only those which were able to induce a systemic activation of the lymphoreticular system (as reflected by splenomegaly) protected against HSV infection. Protection against HSV-infection could also be demonstrated by using killed *Bordetella pertussis*. *C. parvum* also protected against Semliki Forest virus infection in two different strains of mice.

INTRODUCTION

There is increasing evidence to indicate that macrophages play a critical role in the defence against virus infections (Allison, 1974). In certain schedules of infection macrophages appear to represent the first line of defence against the virus. Some viruses such as herpes simplex virus (HSV) are effectively restricted by macrophages which support an abortive infection without replication (Stevens & Cook, 1971). Treatment of mice with silica or anti-macrophage serum has been shown to increase susceptibility to experimental infection with HSV (Zisman *et al.* 1970). Therefore, it is logical to try to combat such virus infections by activating macrophages. Previously, this approach has been little studied but during the completion of our experiments a few reports have appeared demonstrating that certain immunostimulants which are known to cause a general activation of the lymphoreticular system are effective in protecting mice against experimental virus infections (Starr *et al.* 1976; Glasgow *et al.* 1977; Morahan *et al.* 1977). In the present paper we summarize some of our recent data on the protective effect of *Corynebacterium parvum* and *Bordetella pertussis* in HSV-infection of mice.

METHODS

Virus. A strain of HSV-1 designated HSV (WAL) (Kirchner *et al.* 1977*a*) was used in all experiments. This strain, which after repeated passage in mouse brain was highly pathogenic

for mice, was re-adapted to tissue culture and was used after the second passage in human embryo lung cells (HEL). A single stock of virus, frozen in small samples at −70 °C was used in all experiments. It contained 7×10^8 p.f.u./ml when determined by virus plaque assay in cultures of HEL.

Semliki Forest virus (SFV) of the L10 C I strain (Bradish & Allner, 1972) was a gift from Dr C. J. Bradish (Porton Down, Salisbury, U.K.) to Dr V. Schirrmacher. It was passaged once in brains of suckling BALB/c mice.

Mice. Male BALB/c/A BOM, C57BL/6/J BOM, and DBA/2/J BOM mice were obtained from Bomholtgard (Rye, Denmark). They were used in the experiments at the age of 8 to 12 weeks. STU mice which originally had been obtained from Dr W. Schäfer (Tübingen, FRG) were bred in our Department by continuous brother-sister mating.

Bacterial stimulants. Corynebacterium parvum (CN 6134) was a formalin-killed suspension (7 mg dry weight/ml) from Burroughs Wellcome, Beckenham, Kent, U.K. Other strains of anaerobic coryneforms designated CN 5888, CN 6276 and CN 5936 were similarly prepared (Adlam & Scott, 1973) and provided by Dr C. Adlam of Burroughs Wellcome. Dilutions were made in saline.

Killed *Bordetella pertussis* prepared by Behringwerke (Marburg, FRG) was provided through the courtesy of Dr Hof (Würzburg, FRG). It also contained thiomersal and the concentration of bacteria in the original suspension was 10^{11}/ml. It was stored at 4 °C and diluted in saline before use.

Evaluation of animal experiments. Groups of 10 to 20 mice were injected with the immunoadjuvants at various times before or simultaneously with the virus infection. The optimal doses were tested in initial experiments and optimal protection was seen with 350 μg *C. parvum* or 10^8 organisms of *B. pertussis* per mouse. Control groups usually received an injection of saline. Various virus doses were also tested in preliminary experiments and significant protection could often be observed after infection with up to 100 LD_{50}. Routinely, mice were infected with 20 to 40 LD_{50} as indicated in the tables and figures. The time of death was recorded daily for 20 days after virus infection since it was known that no deaths occurred later than 15 days after infection with HSV or SFV. The percentage of surviving mice in the experimental and control group was compared by the chi square test.

Determination of serum levels of interferon. Mice were bled from the retro-orbital sinus and the serum was recovered and diluted in balanced salt solution. The titres of interferon were tested in a plaque-reduction assay using vesicular stomatitis virus and L cells. All details of this assay and of the reference standards used etc. have been described (Hirt *et al.* 1978).

RESULTS

Protection of STU mice by Corynebacterium parvum

In initial experiments we have used STU mice which are quite susceptible to HSV infection, to determine the conditions for protecting mice against viral infection by *Corynebacterium parvum*. Protection was optimal when *C. parvum* was given 7 days before HSV. Little or no protection was seen when it was injected 4 days before, or on the day of virus infection. It was equally effective in protecting STU mice against an intraperitoneal (i.p.) infection with HSV when given by the intravenous (i.v.) route as after i.p. dosage (Table 1).

Table 1. *Protection of STU mice by treatment with killed* Corynebacterium parvum *organisms*

Expt. no.	Treatment	Timing	No. of mice dead/ total no. treated	*P* value
I	*C. parvum** i.p.	7 days before HSV†	4/20	<0·05
	Saline		17/20	
	C. parvum i.p.	3 days before HSV	15/20	NS‡
	Saline		18/20	
II	*C. parvum* i.p.	7 days before HSV	2/20	<0·01
	Saline		18/20	
	C. parvum i.p.	2 days before HSV	17/20	NS
	Saline		20/20	
	C. parvum i.p.	On the day of	16/20	NS
	Saline	HSV infection	17/20	
III	*C parvum* i.v.	7 days before	2/20	<0·01
	Saline	HSV infection§	17/20	

* 350 μg *C. parvum* (CN 6134).
† 5×10^3 p.f.u. (~ 20 LD_{50} after i.p. infection).
‡ Not significant.
§ 2×10^6 p.f.u. (~ 20 LD_{50} after i.v. infection).

Table 2. *Protection of mice of different strains against infection with HSV by* Corynebacterium parvum *and* Bordetella pertussis *given 7 days before infection*

Expt. no.	Strain of mice	Treatment	Dose of HSV	No. of mice dead/ total no. of mice	*P* value
I	DBA/2	*C. parvum*	3×10^4 p.f.u.* i.p.	18/40	< 0·05
		Saline		37/40	
II	BALB/c	*C. parvum*	5×10^5 p.f.u.† i.v.	3/20	< 0·05
		Saline		16/20	
III	C57BL/6	*C. parvum*	5×10^5 p.f.u.‡ i.p.	0/20	< 0·05
		Saline		10/20	
IV	C57BL/6	*C. parvum*	1×10^7 p.f.u. i.p.	3/20	< 0·01
		Saline		18/20	
V	C57BL/6	*C. parvum*	1×10^5 p.f.u. i.p.	2/20	< 0·01
		Saline	+400 R§	17/20	
VI	DBA/2	*B. pertussis* ‖	1×10^5 p.f.u. i.p.	2/20	< 0·01
		Saline		20/20	
VII	C57BL/6	*B. pertussis*	1×10^7 p.f.u. i.p.	0/20	< 0·01
		Saline		18/20	

* 20 LD_{50} in DBA/2 mice after i.p. infection.
† 50 LD_{50} in BALB/c mice after i.v. infection.
‡ The LD_{50} for C57BL/6 mice after i.p. infection with HSV (WAL) is $1{\cdot}2 \times 10^6$ p.f.u.
§ 400 R of X-irradiation on the day of virus infection.
‖ 10^8 killed bacteria were injected 7 days before HSV.

Protection of strains of mice other than STU by Corynebacterium parvum *against HSV infection*

DBA/2 mice could also be protected against i.p. infection with HSV (Table 2). This protection was in most instances less impressive than that observed with STU mice. Clearly, however, the mean number of surviving mice pre-treated with *C. parvum* was significantly higher than that of the controls, pre-treated with saline.

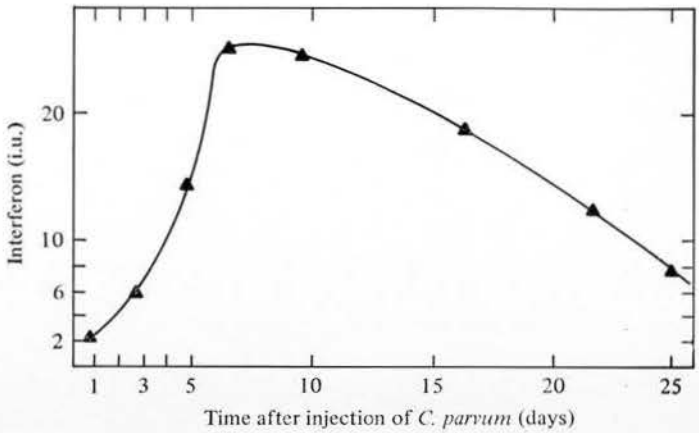

Fig. 1. Serum levels of interferon in C57BL/6 mice at various times after i.p. injection of 350 μg of *Corynebacterium parvum*.

BALB/c mice which, in contrast to other mouse strains, are quite susceptible to i.v. infection with HSV (Kirchner *et al.* 1978) were used to show protection by an i.p. injection of *C. parvum* against this schedule of HSV infection (Table 2).

It was previously shown that C57BL/6 mice, which are relatively resistant to i.p. infection with HSV, could be killed by high virus doses (Kirchner *et al.* 1978). A good protective effect was seen when *C. parvum* was given 7 days before infection of C57BL/6 mice with high doses of HSV. X-irradiation (400 R) when applied on the day of HSV infection to C57BL/6 mice markedly decreased the LD_{50}, but these immunodepressed C57BL/6 mice could still be protected by *C. parvum*, given 7 days before the virus (Table 2).

Protection of DBA/2 mice against HSV infection by Bordetella pertussis

DBA/2 and C57BL/6 mice could be well protected against HSV infection by a previous injection of *B. pertussis* (Table 2). As with *C. parvum*, protection was seen only when the bacteria were injected 7 days before the virus but not when they were given 3 days before injection. The optimal dose for protection was 10^8 organisms per mouse and protection could be observed with doses of HSV up to 100 LD_{50} (data not shown).

Measurement of serum interferon levels

C57BL/6 mice were injected i.p. with 350 μg *C. parvum* and serum interferon levels were determined at various times thereafter. As can be seen in Fig. 1, significant levels of interferon could be detected between 5 and 20 days after injection of *C. parvum*. Little or no interferon was found earlier than 5 days.

Testing of different strains of coryneform bacteria

Four different strains of anaerobic coryneforms (selected by Dr C. Adlam for their differing spleen weight stimulating abilities) were provided in coded form and tested blind for their ability to induce splenomegaly and to protect DBA/2 mice against subsequent HSV infection (Table 3). Only the two bacterial strains which were able to increase the spleen weight were protective against HSV infection.

Table 3. *Comparison of the lymphoreticular stimulating ability of various strains of anaerobic coryneforms with their ability to protect DBA/2 mice against HSV infection**

Anaerobic coryneform strain	Spleen index†	No. of mice dead/ total no. of mice	*P* value
CN 6134	8·2	4/20	< 0·01
CN 5888	1·0	20/20	NS‡
CN 6276	1·5	18/20	NS‡
CN 5936	6·8	9/20	< 0·05
Controls injected with saline	1·0	20/20	—

* 350 μg of the appropriate bacterial strain were injected i.p. and 7 days later mice were challenged with 50 LD_{50} of HSV i.p.

† 350 μg of the bacteria were injected i.v. and 14 days later spleens were removed and weighed. Spleen index: ratio of spleen weights of injected mice compared with saline injected mice.

‡ In comparison with the saline group; NS, not significant.

Table 4. *Effect of previous irradiation of C57BL/6 mice on the protection by* Corynebacterium parvum *against HSV infection**

Group†	Treatment: 400 R-irradiation	Treatment: *C. parvum*	Treatment: HSV	No. of mice dead/ total no. of mice
I	—	—	Day 0	10/10
II	—	Day 7	Day 0	0/10
III	Day 10	—	—	0/10
IV	Day 10	—	Day 0	10/10
V	Day 10	Day 7	Day 0	8/10
VI	Day 3	Day 7	Day 0	2/10

* Experimental conditions as in Expt. IV of Table 2.

† The difference between group II and V was significant ($P < 0·05$) while the difference between group II and VI was not.

Table 5. *Protection of C57BL/6 and DBA/2 mice against i.p. infection with Semliki Forest virus**

Treatment	Mouse strain	No. of mice dead/ total no. of mice	*P* value
Corynebacterium parvum†	C57BL/6	1/20	0·01
Saline		20/20	
C. parvum	DBA/2	2/20	0·01
Saline		19/20	

* Mice were challenged with 100 LD_{50} of SFV.

† 350 μg *C. parvum* per mouse, 7 days before infection.

Effect of previous X-irradiation on the protective effect of Corynebacterium parvum *on HSV infection*

X-irradiation (400 R) given 4 days before *C. parvum* abolished the protective effect of *C. parvum* on HSV infection of C57BL/6 mice (Table 4). However, no such adverse effect was seen when the radiation dose was applied 3 days after *C. parvum* (i.e. 4 days before virus infection).

Protection of C57BL/6 and DBA/2 mice against SFV by injection of Corynebacterium parvum

In contrast to an i.p. infection with HSV (Lopez, 1975) C57BL/6 and DBA/2 mice were found to be equally susceptible to i.p. infection with SFV (Table 5). Both strains of mice could be well protected by injection of *C. parvum* given 7 days before the virus.

DISCUSSION

The genetic resistance of mice to mouse hepatitis virus is determined by a difference in the ability of macrophages from different mouse strains to replicate this virus (Bang & Warwick, 1960). Genetic differences in the susceptibility of mice to HSV infection have been also reported (Lopez, 1975). However, thus far there is little indication that HSV replicates in macrophages (Stevens & Cook, 1971; Kirchner *et al.* 1976) and other reasons have to be sought to explain the genetic resistance of certain mouse strains to HSV. In this regard, it seems to be of interest that susceptible strains of mice could be equally well protected against HSV infection by *Corynebacterium parvum* as C57BL/6 mice which are relatively resistant.

The basis of the protective effect of *C. parvum* on a virus infection has not been fully understood. However, protection is obviously more than just a local effect at the injection site since protection was also seen when *C. parvum* was given by a route other than that of virus infection. *C. parvum* has been shown to induce a strong systemic activation of the lymphoreticular system (Scott, 1974) which appears to be caused by mobilization of macrophage precursors from the bone marrow into the periphery (Baum & Breese, 1976). Not all strains of coryneforms are active in this regard and it is noteworthy that those which were inactive (i.e. did not induce splenomegaly) also caused no antiviral protection. Woodruff *et al.* (1976) have shown that the lymphostimulatory effects of *C. parvum* in mice could be prevented by previous X-irradiation. They have suggested that the effect of *C. parvum* on the macrophage system is, to a considerable extent, due to the stimulation of radiosensitive macrophage precursors to differentiate into mature cells which are actively phagocytic and cytotoxic. Our data are in agreement with their findings since we have observed that the antiviral effects of *C. parvum* could be abolished by previous irradiation.

Previously, it has been shown that mice injected with *Corynebacterium acnes* (which is in most properties closely related to *C. parvum*) displayed a reduced capacity to respond to interferon inducers (Farber & Glasgow, 1972). However, we have found that spleen cells of mice injected *in vitro* with *C. parvum* elaborated increased levels of interferon (Kirchner *et al.* 1977*b*). Here we have found that significant levels of interferon could also be found in the serum of *C. parvum*-treated mice.

The data from Glasgow's laboratory and ours are not necessarily at variance with each other. The interferon produced by spleen cells from *C. parvum*-injected mice is produced only 5 to 10 days after injection. This markedly contrasts with conventional induction of interferon, such as performed by Farber & Glasgow (1972), where high levels of interferon are detected in the serum several hours after injection of the inducer. In interferon research it is known that 'blocking' occurs when cells which are responding to one type of interferon inducer are challenged with a second inducer (Ho & Armstrong, 1975). This may occur when the cells of the lymphoreticular system which are already responding to *C. parvum* are challenged with another inducer.

It is clear, however, that at the present stage we cannot prove that induction of interferon is the cause of the antiviral effect of *C. parvum*. Furthermore, although it is quite likely that

macrophages play a significant role in this protection, their definitive role also remains to be determined.

Bordetella pertussis is known to induce a lymphocytosis in mice (Morse & Riester, 1967) but its effects as an activator of macrophages have been less well studied. In experimental tumour work, divergent results have been reported. In some studies *B. pertussis* was able to protect against a tumour (Likhite, 1974), whereas in others there was little protection (Purnell *et al.* 1975) or even an adverse effect (Floersheim, 1967). In our studies, *B. pertussis* also protected mice against HSV infection, and protection was observed against high virus doses and in a mouse strain where protection by *Corynebacterium parvum* was quite weak. The basis for this protective effect of *B. pertussis* needs to be clarified by further experiments, but it seems of interest that *B. pertussis* also has been shown to be an interferon inducer (Borecky & Lackovic, 1967).

Lately, a number of immunosuppressive effects of the so-called 'immunostimulants' have been noted (Scott, 1972; Kirchner *et al.* 1975). The term 'immunomodulant' has been proposed to indicate that these agents may act on a complicated balance between immunostimulation and immunosuppression. From these observations it seems reasonable to conclude that all of these agents should be handled with care since they may be potentially immunosuppressive. However, it remains to be determined which of these various immune functions contribute to host defence. Protection against a virus infection seems to represent true immunopotentation. *Corynebacterium parvum* is being considered for immunotherapy of human tumours. Since virus infections are a common cause of complications in tumour patients, the antiviral effects of *C. parvum* may be of great clinical significance.

We thank Mr D. Baumgartl for his co-operation in the animal experiments and Ms B. Mannes for excellent secretarial assistance. Semliki Forest virus was a gift from Dr C. J. Bradish and *Bordetella pertussis* was kindly provided by Dr H. Hof, University of Würzburg (FRG).

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BIOLOGICAL EFFECTS OF *CORYNEBACTERIUM PARVUM*: III. AMPLIFICATION OF RESISTANCE AND IMPAIRMENT OF ACTIVE IMMUNITY TO MURINE TUMOURS

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Summary.—The effect of pre-treatment with *Corynebacterium parvum* on the growth *in vivo* of a range of experimental mouse tumours with differing characteristics has been investigated. Varying degrees of protection were observed which were generally greater with the more immunogenic tumours. Administration of *C. parvum* 7 days before immunization with irradiated tumour cells diminished the protective effect which could be obtained by immunization alone. The possible basis for these seemingly conflicting influences is considered.

HALPERN *et al.* (1964) showed that a killed suspension of *Corynebacterium parvum* was an unusually potent stimulant for the reticulo-endothelial (macrophage) system. A number of associated effects such as adjuvant activity (Neveu, Branellec and Biozzi, 1964; Biozzi *et al.*, 1966) and increased resistance to bacterial (Adlam, Broughton and Scott, 1972) and protozoal (Nussenzweig, 1967) infection, have since been reported. The inhibitory effect of *C. parvum* pre-treatment on the growth of a range of experimental mouse tumours (Sarcoma J, Ehrlich ascites, a spontaneous mammary carcinoma, a methylcholanthrene-induced sarcoma and the AKR leukaemia) has been described (Halpern *et al.*, 1966; Woodruff and Boak, 1966; Lamensans *et al.*, 1968). Although the results obtained here varied between the different systems, in general, conditions could be found under which *C. parvum* afforded a degree of protection. Currie and Bagshawe (1970) used a combination of *C. parvum* and chemotherapy against a methylcholanthrene-induced fibrosarcoma with some success, whereas Mathé, Pouillart and Lapeyraque (1969) were unable to influence an established L1210 tumour with a combination of *C. parvum* and immunotherapy.

The experiments described here are concerned with two aspects of the anti-tumour activities of *C. parvum*: the effect of simple pre-treatment and of pre-treatment followed by active immunization on the growth of a primary tumour challenge. Several mouse tumour systems, providing a variety of growth patterns and immunogenicities, have been used and the relationship between the effects of *C. parvum* and the characteristics of the tumours are discussed. The investigation formed a prelude to studies at the cell level and tissue culture lines have been established from the tumours described, in order to facilitate subsequent work *in vitro*.

MATERIALS AND METHODS

Mice.—Adults of the following inbred strains and F_1 hybrids, maintained in this Department, were used: CBA-*p* (from the Department of Genetics, University of Cambridge), DBA/2 and BALB/c (from the Chester Beatty Research Institute) and (BALB/c × DBA/2)F_1. Groups of 10 mice were used for each experimental group throughout.

Corynebacterium parvum.—A killed suspension of *C. parvum* (Batch No. EZ174 7 mg/

ml) was provided by Wellcome Research Laboratories, Beckenham, Kent, England. A standard dose of 0·2 ml (1·4 mg) was injected i.v. or i.p.

Tumours.—The following tumours were obtained from the Chester Beatty Research Institute and maintained in ascitic form in the mice specified: R–I (radiation induced CBA leukaemia; Hewitt, 1962) in CBA-*p*; Hepatoma 129 (induced by CCl_3 in a C3H mouse, Andervont and Dunn, 1955) in CBA-*p* and BALB/c; Adj. PC6A (adjuvant induced BALB/c plasmacytoma, Potter and Robertson, 1960) in BALB/c and L5178 (DBA/2 leukaemia, Fischer, 1958) in (BALB/c × DBA/2)F_1.

The CBAT-3 fibrosarcoma was derived originally from a tissue culture line of CBA embryo fibroblasts and maintained as a subcutaneous solid tumour in CBA-*p* mice.

For both routine passage and experimental use, tumours were handled as cell suspensions in phosphate buffered saline. Ascites cells were harvested direct and washed once. Solid tumours were dissociated by mincing with scissors and pipetting the fragments. The supernatant cells, after settling, were washed once.

Inactivation of tumour cells.—Cells were inactivated by *in vitro* irradiation (15,000 rad) from a ^{60}Co source. This procedure did not affect the viability of the cells as judged by trypan blue exclusion.

Assessment of results.—Results are displayed as curves of percentage of survivors against days after challenge. Animals whose deaths are not recorded were kept for 60 days. In the case of the subcutaneous CBAT-3 tumour, a 2 cm diameter was taken as the endpoint if it occurred before death.

RESULTS

Characteristics of the tumours and their immunogenicity

Immunogenicity of the various tumours was assessed by the protective effect of immunization with inactivated tumour cells 10 days before challenge with normal tumour cells. Immunization against ascitic tumours was i.p., but for solid tumours the immunizing dose was split between the i.p. route and a subcutaneous (s.c.) site contralateral to that of the challenge.

RI leukaemia cells are syngeneic in CBA mice and grow rapidly, causing death from inocula of fewer than 10 cells. Death is abrupt with only moderate ascites development, suggesting that metastasis is a major factor. The tumour as first grown here was moderately immunogenic in CBA-*p* mice, a single dose of 10^5–5×10^5 irradiated cells giving 50% protection against a 100 cell challenge. This form of the tumour is referred to as RI. Weekly passage through mice for 18 months resulted in a variant form which was very much less immunogenic (RI-var.); 5×10^5 irradiated RI-var. gave almost no protection against a 100 cell challenge. Recourse to frozen stored material allowed comparisons between the original and variant forms.

Hepatoma 129 grows rapidly from inocula of less than 10 cells and causes death with gross ascites and no evidence of metastases. Our form is not strain specific; originally a C3H tumour, it is routinely passaged in CBA against a minor histocompatibility barrier and a secondary strain is passaged in BALB/c mice against a strong histocompatibility barrier. There is little difference between the growth rate or minimum lethal dose of the two strains. The immunogenicity of irradiated cells is also similar, a dose of between 10^4 and 5×10^4 giving 50% protection against a 100 cell challenge. The lack of strain specificity is not due to masking or loss of histocompatibility antigens, since BALB/c mice were readily immunized against the tumour by normal CBA spleen cells.

CBAT-3 fibrosarcoma grows as a non-metastasizing solid when injected s.c. Its liability to become haemorrhagic or to break through the skin makes quantitative assessment difficult. When injected i.p. it grows more rapidly as localized solid tumours in the peritoneal wall and consistently causes death in the animals.

Assessment of the immunogenicity of irradiated cells depends on the route of injection of challenge cells. Protection

is low against s.c. challenge with 10^4 cells, 10^6–5×10^6 irradiated cells being required to give 50% protection. The titration with i.p. challenge did not reach an endpoint but protection was at least 10 times greater.

L5178 leukaemia causes death essentially by massive i.p. growth with some local infiltration to form solid mesenterial growth. The F_1 hybrid in which the tumour was passaged was within the major histocompatibility group ($H\text{-}2^d$) of the strain of origin (DBA/2), thus minimizing any potential effects of allogeneic inhibition. The tumour was poorly immunogenic in this system and up to 10^7 irradiated cells were required for 50% protection against a 100 cell challenge.

PC6 plasmacytoma gives rise to massive ascites, with ready formation of a solid tumour at the injection site; it shows no evidence of metastasis. It is poorly immunogenic, with no protection against a 10^3 cell challenge with irradiated cell doses of up to 10^7.

Protective effects of pre-treatment with C. parvum

Groups of 10 mice received 0·2 ml of *C. parvum* i.v. or i.p. 7 days before challenge with tumour cells. When *C. parvum* is administered 7 days before injection of sheep red cells it produces a marked adjuvant effect (Scott, unpublished results).

RI leukaemia.—Challenge doses of both 10 and 100 cells were assayed (Fig 1A, B). In all cases there was a definite protective effect from *C. parvum* which was highly significant at the lower challenge dose and with little difference between the i.v. and i.p. routes of administration. It was noticeable that prolongation of survival of animals incompletely protected by *C. parvum* was accompanied by greater ascites development. Very little protection from *C. parvum* was

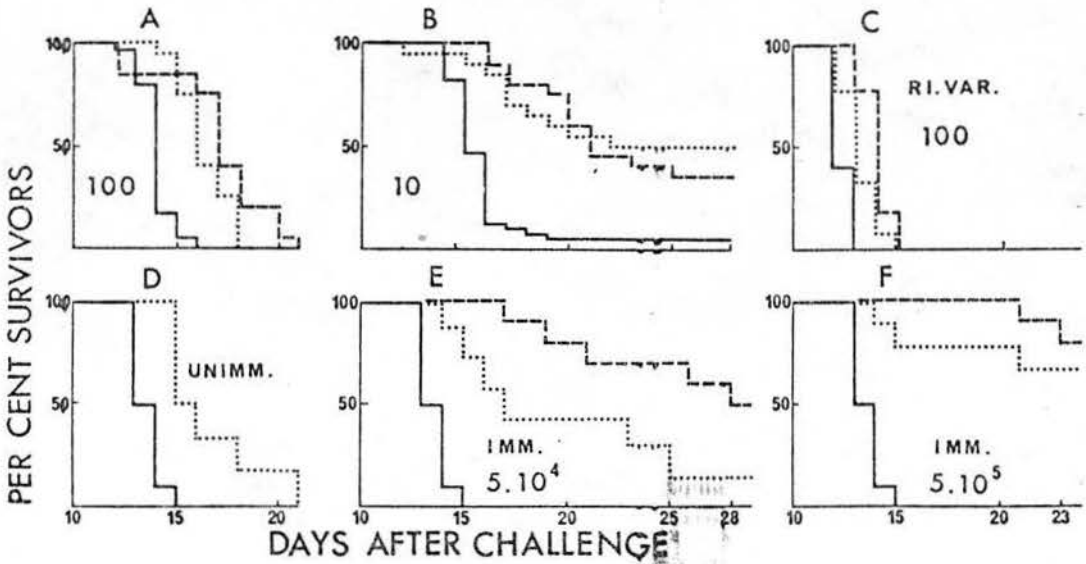

FIG. 1 A–C.—The effect of *C. parvum* pre-treatment on RI leukaemia in CBA mice. 1·4 mg *C. parvum* 7 days before challenge with 100 RI (a), 10 RI (b) and 100 RI-variant (c) leukaemia cells. *C. parvum* i.v. (. . . . .), *C. parvum* i.p. (– – – –), none (————).

D–F.—The effect of combined *C. parvum* pre-treatment and immunization on RI leukaemia in CBA mice. 1·4 mg *C. parvum* i.v. 7 days before immunization i.p. with 5.10^4 (e), 5.10^5 (f) irradiated leukaemia cells or none (d). All mice were challenged i.p. with 100 RI leukaemia cells after a further 7 days. Untreated mice (————), immunization alone (– – – –), *C. parvum* + immunization (. . . . .).

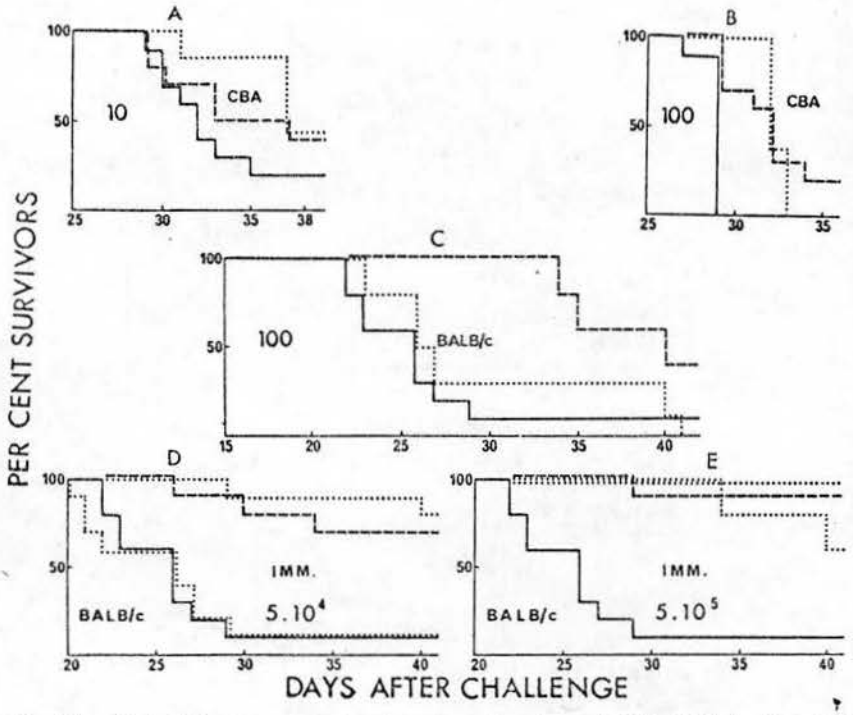

FIG. 2 A–C.—The effect of *C. parvum* pre-treatment on Hepatoma 129 in CBA (a, b) and BALB/c (c) mice. 1·4 mg *C. parvum* 7 days before challenge with 10 or 100 tumour cells. *C. parvum* i.v. (. . . . .), *C. parvum* i.p. (– – – –), none (———).

D–E.—The effect of combined *C. parvum* pre-treatment and immunization on Hepatoma 129 cells in BALB/c mice. 1·4 mg *C. parvum* 7 days before immunization i.p. with 5.10^4 (d), 5.10^5 (e) irradiated tumour cells. Untreated mice (———), immunization alone (+ + +). *C. parvum* i.v. + immunization (. . . . .), *C. parvum* i.p. + immunization (– – – –).

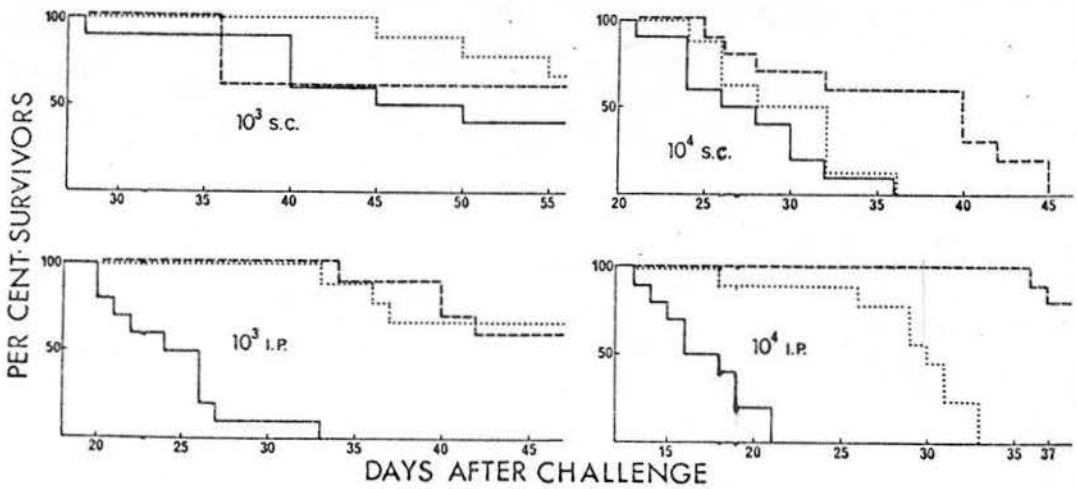

FIG. 3.—The effect of *C. parvum* pre-treatment on CBA–T3 fibrosarcoma in CBA mice. 1·4 mg *C. parvum* 7 days before challenge with either 10^3 or 10^4 tumour cells s.c. (top row) or i.p. (bottom row). *C. parvum* i.v. (. . . . .), *C. parvum* i.p. (– – – –). none (———).

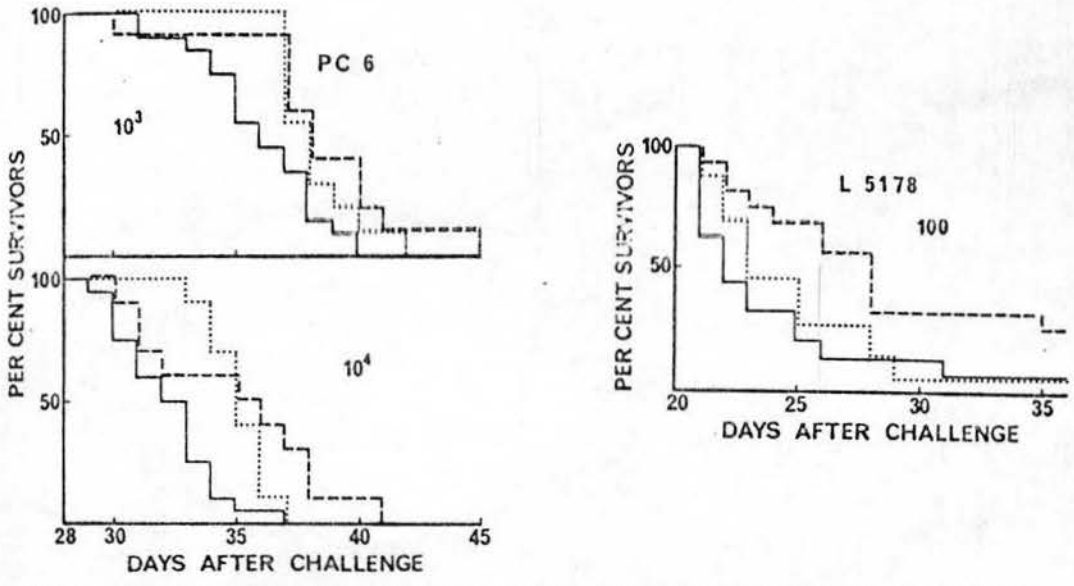

FIG. 4.—The effect of *C. parvum* pre-treatment on PC6 plasmacytoma in BALB/c mice (left) and L5178 leukaemia in (BALB/c × DBA/2)F_1 (right). 1·4 mg *C. parvum* 7 days before challenge i.p. with 10^3 or 10^4 PC6, or 100 L5178 cells. *C. parvum* i.v. (.....), *C. parvum* i.p. (– – – –), none (———).

afforded against the poorly immunogenic RI-var. as compared with the original RI (Fig. 1C).

Hepatoma 129.—Challenges of both 10 and 100 cells in the CBA system and 100 cells in the BALB/c system were assayed (Fig. 2A–C). In CBA mice the protective effect was small, with little difference between *C. parvum* i.v. and i.p. The situation was quite different with BALB/c mice: whereas *C. parvum* i.v. had little effect, there was a strong protective effect following i.p. administration.

CBAT-3 *fibrosarcoma*.—Both s.c. and i.p. challenge with 10^3 and 10^4 cells were investigated (Fig. 3): s.c. challenge with 10^3 cells proved too small for analysable results, although *C. parvum* pre-treatment both i.p. and i.v. increased the number of survivors. At the 10^4 dose there was a definite effect with *C. parvum* i.p. but very little with i.v. injection. With i.p. challenge of 10^3 cells both i.v. and i.p. *C. parvum* afforded strong protection. This protection was maintained by i.p. *C. parvum* against a 10^4 cell challenge, but the i.v. route was again less effective.

L5178 leukaemia.—The effect of *C. parvum* pre-treatment against a 100 cell challenge was minimal (Fig. 4).

PC6 plasmacytoma.—Pre-treatment again afforded only slight protection against challenges of 10^3 and 10^4 cells (Fig. 4).

Combined pre-treatment with C. parvum and irradiated cells

C. parvum was injected 7 days before immunization with irradiated cells and the animals were challenged after a further 7 days. Groups of 10 mice were used.

RI leukaemia.—The results of combining immunization by 5×10^4 or 5×10^5 irradiated cells with i.v. *C. parvum* are shown in Fig. 1D–F. *C. parvum* pre-treatment depressed the protective effect of immunization, more especially with the lower immunizing dose, but exerted its normal protective effect when given alone.

Hepatoma 129.—The outcome of combining immunization with *C. parvum* pre-treatment was investigated in the BALB/c system. Two immunizing doses

TABLE I.—*The Protective Effect of C. parvum Pre-treatment Against Experimental Mouse Tumours of Varying Immunogenicity*

Tumour and strain of origin	Tumour type	Experimental host and challenge route	Immunogenicity*	*C. parvum* protection†	
				i.v.	i.p.
RI CBA	Ascites leukaemia	CBA i.p.	××	++	++
RI-var. CBA	Fibrosarcoma	CBA i.p.	⩽×	±	±
H129 C3H	Ascites hepatoma	CBA i.p.	×××	+	+
H129 C3H	Ascites hepatoma	BALB/c i.p.	×××	±	+++
L5178 DBA/2	Ascites leukaemia	(BALB/c×DBA/2)F_1 i.p.	×	±	+
PC6 BALB/c	Ascites plasmacytoma	BALB/c i.p.	<×	±	±
CBAT-3 CBA	Fibrosarcoma	CBA s.c.	×	+	+
CBAT-3 CBA	Fibrosarcoma	CBA i.p.	⩾××	++	+++

* Graded according to dose of irradiated tumour cells giving 50% protection against a challenge of 10–20 times the LD_{50} of living tumour cells: ××× 10^4–10^5; ×× 10^5–10^6; × 10^6–10^7.

† Subjective grading from good (+++) to poor (±) protection.

(5×10^4 and 5×10^5) were used with both i.v. and i.p. *C. parvum* (Fig. 2D–E). Good protection was induced with i.p. *C. parvum* alone and such treatment did not modify the protective effect of immunization. Administration of *C. parvum* i.v., which alone afforded minimal protection, completely abolished the protective effect of the lower immunizing dose of irradiated cells and slightly reduced that of the high dose.

DISCUSSION

C. parvum pre-treatment was found to confer some degree of protection against a variety of highly adapted and rapidly growing mouse tumours, in agreement with reports by previous workers (Halpern *et al.*, 1966; Woodruff and Boak, 1966; Lamensans *et al.*, 1968).

It seemed likely that *C. parvum* would be more effective against the more immunogenic tumours and such a correlation is seen in the comparison of the normal and variant RI strains. The variation in results obtained with the different tumour systems serves to emphasize the complexity of the mechanisms involved, but the more striking effects were found in the immunologically reactive situations (Table I). *C. parvum* causes intense reticulo-endothelial stimulation (Halpern *et al.*, 1964) and stimulated macrophages have been reported to become non-specifically cytotoxic to tumour cells (Alexander and Evans, 1971; Hibbs, Lambert and Remington, 1972). Such effects may explain, for example, the slight protection against the very poorly immunogenic PC6 tumour, but the suggested correlation between immunogenicity and *C. parvum* protection implies involvement of specific immunological defences in addition to any non-specific activation.

The i.p. route for *C. parvum* was somewhat more effective than the i.v. although both routes were similar with respect to spleen and liver enlargement and neither led to an increased peritoneal cell population at the time of challenge (authors' unpublished observations). That the difference cannot be explained simply by local i.p. effects is also shown by the greater efficacy of i.p. *C. parvum* in the s.c. CBAT-3 system.

In view of the known adjuvant activity of *C. parvum*, it was anticipated that combination with immunization would result in increased protection compared with the latter alone. However, in both the systems investigated, i.v.

C. parvum, given before immunization, decreased the protective effect, although i.p. *C. parvum* in the H129 system did not influence the immune response. It seems that while *C. parvum* treatment stimulates the defences of the host against many tumours it may also, at least when given i.v., concomitantly depress some part of the immune response. Preliminary experiments both *in vitro* and *in vivo* have so far failed to reveal evidence for production of enhancing antibody and the fact that the depression can be overridden by larger immunizing doses also argues against such a mechanism. Enhanced growth of allogeneic tumour cells following immunization in conjunction with either complete or incomplete Freund's adjuvant has recently been reported (Zola, 1972), and again no evidence of enhancing antibody could be found, the effect being attributed to depression of cell-mediated cytotoxicity. Evidence of depressed cell-mediated (T lymphocyte) responses following *C. parvum* treatment comes from the demonstration of reduced delayed hypersensitivity (Asherson and Allwood, 1971), PHA responsiveness and GVH reactivity of lymphocytes (Scott, 1972).

Schedules for the immunotherapeutic treatment of human tumours have often included adjuvants, and *C. parvum* has begun to figure among these (Mathé, 1971). The present finding that this agent, at least under certain experimental conditions, can show an immunosuppressive component in its action urges caution in this field.

We wish to acknowledge the able technical assistance of Mrs S. Wishart and Miss C. Tieman. Thanks are also due to Dr J. G. Howard for helpful criticism.

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Corynebacterium parvum as a Therapeutic Antitumor Agent in Mice. I. Systemic Effects From Intravenous Injection [1,2]

Martin T. Scott [3,4]

SUMMARY—Two days after mastocytoma P815 was established as a solid tumor in the foot pads of (C57BL/6 × DBA/2)F_1 mice, intravenous (iv) injection of *Corynebacterium parvum* caused only transient inhibition of tumor growth with no complete regressions. Tumor inhibition was reflected in increased survival times. Some toxicity problems were encountered after the high doses required for maximum effect were given, and multiple doses of *C. parvum* were no more effective than single doses. The antitumor effects of *C. parvum* were still apparent in T-cell-deprived mice. Mice treated iv with *C. parvum* did not develop systemic antitumor immunity, but peritoneal macrophages and spleen cells from *C. parvum*-stimulated animals were nonspecifically cytostatic for mastocytoma cells in vitro. Thus systemic *C. parvum* was predominantly nonspecific in its mode of antitumor action.—J Natl Cancer Inst 53:855–860, 1974.

THE ANTITUMOR ACTIVITY of killed *Corynebacterium parvum* in mice, rats, and humans has been described (*1–4*). Various routes of administration have been used; this paper describes some aspects of the use of systemically administered *C. parvum* given intravenously (iv) as a therapeutic antitumor agent in mice. The companion paper describes the use of locally administered *C. parvum* in the same tumor model (*5*). Systemic injection of *C. parvum* markedly stimulates the reticuloendothelial system, as evidenced by splenomegaly, hepatomegaly, and increased carbon clearance; all are attributable to marcophage activation (*6, 7*). Consideration of previous indirect evidence led to the deduction that this nonspecific marcophage stimulation, rather than any augmentation of specific antitumor responses (*8*), underlies the antitumor effects of systemically administered *C. parvum*.

MATERIALS AND METHODS

Mice.—Adult (C57BL/6 x DBA/2)F_1 mice (hereafter designated B6D2F_1) were used.

T-cell-deprived mice.—Four- to six-week-old mice were surgically thymectomized and, 6 days later, given 900 rads whole-body irradiation. The next day they received 5×10^6 syngeneic bone marrow cells iv and were used for experimentation 5 weeks after reconstitution.

C. parvum.—A formol-killed suspension of *C. parvum* (batch #PX289, 7 mg/ml) was obtained from Wellcome Research Laboratories, Beckenham, Kent, England. It was diluted with saline.

Tumor.—The mastocytoma P815 (MA) was originally induced by 3-methylcholanthrene in a DBA/2 mouse. It was obtained in the frozen state from Dr. Virginia Evans, Tissue Culture Section, National Cancer Institute, Bethesda, Maryland, and has been maintained in my laboratory by serial ascites passage in B6D2F_1 mice for 18 months. Experimental cells were obtained by peritoneal lavage with Hanks' balanced salt solution (HBSS) containing 20 U heparin/ml; all dilutions and injections were made with this medium. MA P815 is poorly immunogenic in B6D2F_1 mice [(*9*) and these experiments]. Injection of 10^7 irradiated cells iv 7 days before subcutaneous (sc) injection of 10^6 live cells did not affect the growth of the solid tumor.

Irradiated tumor cells.—Seven days after they received 10^6 MA cells intraperitoneally (ip), mice were irradiated with 4000 rads immediately before peritoneal lavage.

In vitro assay for tumor cytostatic activity of C. parvum-activated cells.—Peritoneal cells were obtained by lavage with HBSS containing 10 U heparin/ml. They were washed and resuspended in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Spleen cells were prepared by gently pushing spleens through a fine stainless-steel sieve; they were washed, filtered, and resuspended in MEM with 10% FCS. Target tumor cells were obtained as described above and resuspended in MEM with 10% FCS. One ml of the appropriate concentration of effector cells was added to 1 ml (10^5) tumor cells in 16×125-mm tissue culture tubes (Falcon Plastics, Los Angeles, Calif.). Cultures were incubated at 37° C in a 5% CO_2 atmosphere. After 24 hours of culture, 1 μCi tritiated thymidine (New England Nuclear Corp., Boston, Mass.) was added to each tube; 16 hours later, isotope incorporation into the acid-precipitable material was assayed as described in (*10*) except that Whatman glass fiber papers (2.5 cm G F/B) (H. Reeve Angel and Co. Ltd., London, England) were used. All cultures were set up in quadruplicate.

RESULTS

Tumor Growth

MA cells (10^6) were routinely injected sc into a hind foot pad in 0.05 ml HBSS; 30-gauge needles were used. Subsequent tumor growth in the foot pad was measured as foot-pad thickness, in 0.1-mm units, with a Schnelltaster dial gauge caliper A02T (H.C. Kroplin, Schluctern, Hassen, West Germany). The suitability of the foot pad as a site of growth for this

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[3] Trudeau Institute, Inc., P.O. Box 59, Saranac Lake, N.Y. 12983.

[4] The excellent technical assistance of Sandra L. Warner is gratefully acknowledged.

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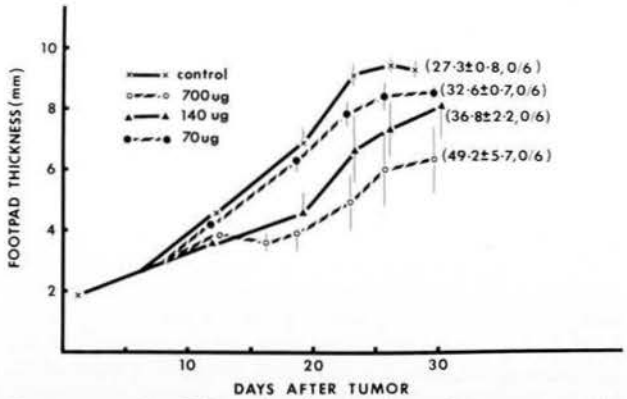
particular tumor and the method of assessing tumor growth has been described (*9*).

In control mice, tumors became evident after about 1 week and progressed until a foot-pad thickness of 8-10 mm was attained, usually after 25–30 days. The tumor consistently killed control mice; the survival time for the whole experimental series was 29.5± 1.3 days (mean ± SE). During this period, tumor growth was generally restricted to the primary site; however, in mice that survived with primary tumors of 9–10 mm, tumor growth in the legs and enlargement of the inguinal nodes became apparent. This was accompanied by necrosis of the primary tumor and eventual loss of the foot. Once such necrosis was evident, measurements were discontinued and the mice were scored only for survival. The tumors metastasized; occasional lesions were found at autopsy in the liver, kidneys, and spleen. Data from from repeated experiments showed that, depite the occasional reduction in group size due to death or foot loss, the mean tumor size over the period when growth was restricted to the primary site provided a consistent and reliable measure of the difference in rate of tumor growth between control and treated animals. The differences in tumor size between the various groups at each time point were analyzed statistically and relevant differences are noted in the text.

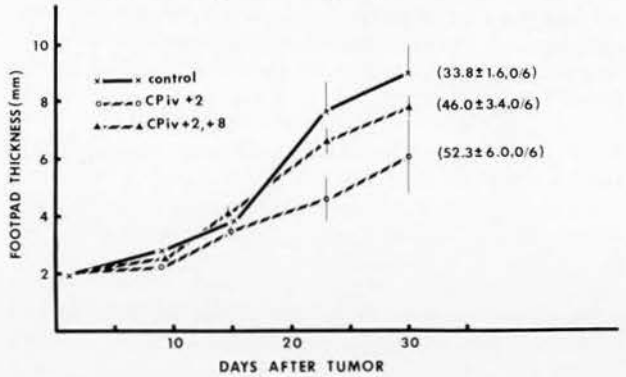
iv *C. parvum* Therapy

In the first series of experiments, groups of mice received 10^6 MA cells sc in the right hind foot pads and were treated iv 2 days later with 0.1 ml of various doses of *C. parvum*. The optimal dose for inhibition of tumor growth was 700 μg; higher doses (1400 μg) resulted in some deaths due to toxicity,[5] and lower doses were less effective in inhibiting tumor growth (text-fig. 1). The effect of *C. parvum* became manifest between 13 and 15 days and lasted about 8 days, whereupon tumor growth was again apparent and continued at a rate similar to that in untreated controls. The protective effect of *C. parvum* was also reflected in the markedly increased survival times; however, in this and repeat experiments no complete regressions occurred. Experiments in which treatment was delayed beyond 2 days resulted in reduced inhibition of tumor growth. Continued observation of mice given *C. parvum* iv showed that they could tolerate a much larger tumor mass than their untreated counterparts. The primary tumor in the foot grew to 8–10 mm (when all untreated animals died) and became necrotic; the foot eventually dropped off and the tumor continued to grow in the upper region of the leg.

The time at which the antitumor effect of *C. parvum* became apparent (13–15 days) is coincident with the "maximum stimulation" of animals after iv treatment with *C. parvum*, as evidenced by splenomegaly and hepatomegaly (*7*). In a second series of experiments, a second iv dose of *C. parvum* was given 6 days after the first and was timed to prevent the escape from the influence of *C. parvum* seen at ≈21 days. Text-figure 2 shows that the effect of the multiple *C. parvum* dose on



TEXT-FIGURE 1.—Effect of various iv doses of *C. parvum* on the growth rate of 10^6 MA cells injected into a hind foot pad 2 days earlier. *Numbers in parentheses* represent mean survival time ± SE of those animals that died, and number of survivors/No. in experimental group.



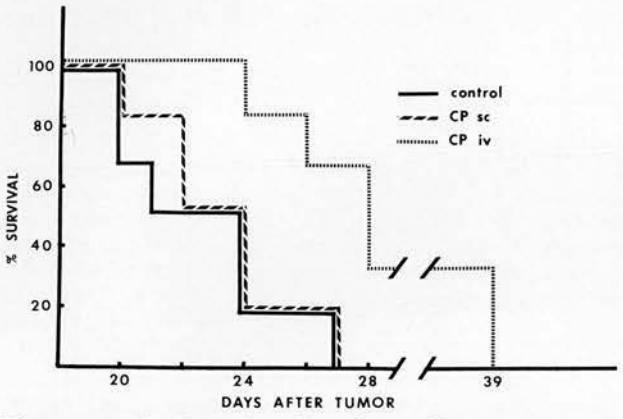
TEXT-FIGURE 2.—Effect of multiple iv doses of *C. parvum* (CP) on the growth rate of 10^6 MA cells growing in the foot pad. Mice received 700 μg CP iv 2 days after tumor establishment (O▼▰▲O), 2 and 8 days after tumor (▲▼▰▲▲), or after no treatment (X▬▬X). *Numbers in parentheses* as for text-figure 1.

tumor growth rate was apparently not as good as the single dose; however, this was only significantly different at day 23 ($P<0.05$). Overall survival times were not significantly different. In some experiments, the mice did not tolerate the multiple high doses of *C. parvum* well (*see* footnote 5).

Effect of *C. parvum* Given iv Against iv Tu mor Challenge

Injection of low doses of MA (10^6) iv killed all normal mice; the survival time was 20.3±1.5 days (mean ± SE). At autopsy, tumor nodules, often 1–2 mm in size, were found in both the liver and kidneys. To determine any effect of systemic *C. parvum* on tumor cells disseminated via the blood, mice received *C. parvum* iv and were challenged 14 days later with 10^4 live MA given iv; survival times were noted (text-fig. 3). There was a marked increase in survival time after *C. parvum* treatment iv; however, mice that received *C. parvum* sc into the neck (i.e., no systemic

[5] Deaths due to toxicity were about 5% with a dose of 1400 μg and invariably occurred within 24 hours of treatment. These deaths were therefore easy to differentiate from deaths resulting from tumor.

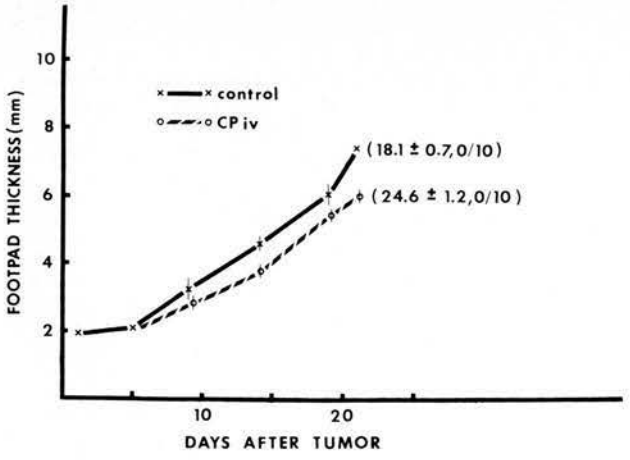


TEXT-FIGURE 3.—Protective effect of either 700 μg *C. parvum* iv or sc into the neck against 10^4 MA cells iv 14 days later. Control mice were untreated; groups of 8 animals were used.

stimulation) survived only as long as untreated controls. The survival times of the controls and groups given *C. parvum* iv were 22.7 ± 1.2 (mean $\pm$ SE) and 30.7 ± 2.7 days, respectively ($P < 0.025$).

Antitumor Effect of *C. parvum* Given iv in T-Cell-Deprived Mice

The growth characteristics of 10^6 MA in the foot pads of T-cell-deprived mice were similar to those of intact mice, but survival times were consistently shorter. Text-figure 4 shows the effect of iv *C. parvum* injections 2 days after tumors were established in the foot. The usual marked inhibition of tumor growth seen around 14 days in intact mice after iv *C. parvum* administration (700 μg) (text-fig. 1) was absent; however, the tumors in the treated mice were significantly smaller than in the controls from day 12 on. The increased survival time of treated mice was also significant ($P = 0.001$). This represented a 35.9 ± 6.6 (SE) % increase in survival time over untreated controls. The comparable figure for intact mice so treated was $49.3 \pm 3.0\%$. This latter figure was based on retrospective data accumulated throughout the whole experimental series; however, given this limitation, the respective increases in survival time were not significantly different.



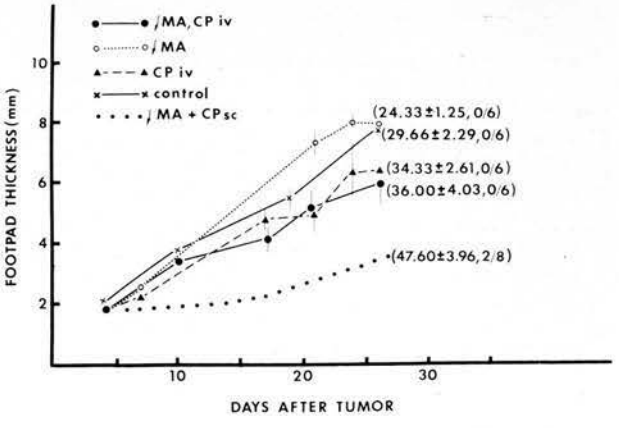
TEXT-FIGURE 4.—Effect of *C. parvum* given iv on the growth of 10^6 MA cells injected into the hind food pads of T-cell-deprived mice. *C. parvum* (700 μg) was injected 2 days after the tumor cells. Control T-cell-deprived mice received no treatment. *Numbers in parentheses* as for text-figure 1.

Immune Status After iv *C. parvum* Treatment

The model devised to demonstrate any systemic antitumor immunity following iv *C. parvum* treatment was as follows: Mice received 10^7 irradiated MA (4000 rads) cells sc into a hind foot pad. One day later, *C. parvum* was given iv. Seven days after *C. parvum* treatment, the mice were challenged with 10^6 live MA in the contralateral foot pad, and growth of this tumor was monitored for signs of systemic immunity (text-fig. 5). Injection of irradiated MA cells alone resulted in a marginal (not significant) increase in tumor growth rate, and iv administration of *C. parvum* alone caused its expected inhibition of tumor growth with concomitantly increased survival times. The combination of irradiated cells followed by *C. parvum* injected in was no more effective than *C. parvum* given alone iv; thus no systemic antitumor immunity had developed. Included in the graph is a positive control showing that such a system can detect immunity when the irradiated cells and *C. parvum* are injected sc at the same site. Injection of *C. parvum* sc alone had no effect.

In Vitro Evidence for Nonspecific Antitumor Activity

Noninduced peritoneal leukocytes from mice that had received 700 μg *C. parvum* iv 7 days earlier inhibited proliferation of MA cells when cultured with them in vitro at the ratio of 10:1 (table 1). Normal peritoneal leukocytes were, however, without effect. Cytostatic activity was more marked if the *C. parvum* was injected ip and peritoneal exudate cells were used 4 days later, but thioglycolate-induced peritoneal exudate cells were without effect (table 1). To characterize the peritoneal leukocytes involved (table



TEXT-FIGURE 5.—Effect of foot-pad injection of 10^7 irradiated MA cells followed 1 day later by iv *C. parvum* (700 μg) (●—●) on the growth of 10^6 live MA cells injected into the contralateral hind foot 7 days later. Pretreatment for other groups was irradiated MA alone (○ - - ○), *C. parvum* iv alone (▲—▲), or 10^7 irradiated MA+175 μg *C. parvum* into the hind foot pad (• • • •). Control mice were untreated (X—X). *Numbers in parentheses* as for text-figure 1.

2), *C. parvum*-activated peritoneal cells were irradiated in vivo with 4000 rads immediately before culture with MA cells. As shown, the irradiation did not reduce the antitumor activity. Further characterization was achieved by allowing the *C. parvum*-activated peritoneal cells to settle in the plastic tube for 3 hours at 37° C and then thoroughly washing away the nonadherent cells. MA cells were added to the MA-adherent cells and assayed for proliferation as before. The cytostatic activity of the adherent cells was equivalent to that of whole peritoneal exudates, indicating that the antitumor activity resides in this population. Histologic examination of the adherent cells showed them to be more than 80% macrophages.

I tried to demonstrate similar activity of spleen cells after iv treatment with *C. parvum* by the same assay system. No significant difference between normal and *C. parvum*-stimulated spleen cells was detectable at a ratio of spleen to tumor cells of 10:1, 50:1, or 100:1. At a ratio of 200:1, normal cells markedly inhibited tumor cell proliferation; however, *C. parvum*-activated cells were significantly more inhibitory ($P=0.001$) (table 3).

DISCUSSION

Treatment of mice bearing the poorly immunogenic MA P815 with *C. parvum* iv resulted in a transient inhibition of tumor growth and concomitantly increased survival times. No complete regressions occurred. That a single systemic dose of *C. parvum* can inhibit the growth of previously established solid tumors agrees with the findings of other workers who used mouse and rat tumor models (*1*, *2*). In the present study, high doses of *C. parvum*, limited only by toxicity, were required for the maximum antitumor effect, and this would seem to be substantiated by the fact that unrelated studies have employed similar high doses (*1–3*). Attempts to augment the effect by using multiple doses of *C. parvum* were unsuccessful; however, increased effectiveness of multiple doses of *C. parvum* have been reported with a

TABLE 1.—*In vitro cytostatic activity of C. parvum-activated peritoneal leukocytes for MA cells*

Cells	DNA synthesis (cpm/culture ± SE)	MA alone* (cpm %)
MA alone	33, 776 ± 4737	100. 0
Normal peritoneal cells	435 ± 42	—
" " " + MA (10:1)	39, 945 ± 1800	118. 3
iv *C. parvum* peritoneal cells†	401 ± 51	—
" " " " + MA (10:1)	11, 996 ± 2134	35. 5
ip *C. parvum* peritoneal cells‡	396 ± 48	—
" " " " + MA (10:1)	3109 ± 749	9. 2
Thioglycolate peritoneal cells + MA (10:1)§	37, 363 ± 6574	110. 6

* The cpm/cultures without MA are minimal and not significantly different, and so have been omitted from this calculation.
† Peritoneal washouts from mice that had received 700 μg *C. parvum* iv 7 days earlier.
‡ Peritoneal washouts from mice that had received 700 μg *C. parvum* ip 4 days earlier.
§ Peritoneal washouts from mice that had received 0.5 ml thioglycolate ip (Difco Laboratories Inc., Detroit, Mich.) 4 days earlier.

TABLE 2.—*Characteristics of C. parvum-activated peritoneal leukocytes cytostatic for MA cells*

Cells	DNA synthesis (cpm/culture ± SE)	MA alone (cpm %)
MA alone	72, 094 ± 18, 966	100. 0
ip *C. parvum* peritoneal exudate + MA*	2136 ± 347	11. 6
" " " " 4000 rads + MA†	1218 ± 212	6. 6
" " " " adherent + MA†	5392 ± 276	7. 5

* Peritoneal exudates from mice that had received 700 μg *C. parvum* ip 4 days earlier.
† *See* text.

TABLE 3.—*In vitro cytostatic activity of C. parvum-activated spleen cells for MA cells*

Cells	DNA synthesis (cpm/culture ± SE)	MA alone (cpm %)
Normal spleen cells	4360 ± 126	—
" " " + MA (200:1)	14, 308 ± 1119	27. 8
C. parvum iv spleen cells*	4900 ± 204	—
" " " + MA (200:1)	9528 ± 866	18. 5
MA alone	51, 385 ± 6174	100. 0

mouse mammary carcinoma system (*1*). Any increased effectiveness would have to be balanced against the increased toxicity of such regimens. The toxicity of systemically injected *C. parvum* has been noted in other studies (*11, 12*), and particularly after multiple injections (*13*), where it was considered to have an allergic basis.

No systemic antitumor immunity was detected after systemic administration of *C. parvum;* this suggests that augmentation of specific antitumor responses is not a component of the means by which systemic *C. parvum* effects its antitumor activity. Similar negative results have recently been described with immunogenic tumors in both mice and rats when both *C. parvum* and tumor antigen were administered systemically. Tumor growth in animals given ip injections of *C. parvum* mixed with irradiated tumor cells was the same as in animals receiving *C. parvum* alone (*2*). Other considerations support the idea that systemic *C. parvum* is nonspecific in its antitumor activity. In the present study, severe depletion of T cells did not reduce the overall effect of *C. parvum*, as judged by percent increase in survival time. Woodruff et al. (*14*) have also shown that *C. parvum*-mediated inhibition of tumor growth is comparable in both normal and T-cell-deprived mice. Further experiments with *C. parvum* in high and low antibody responder mice may also argue against a determinant role for antibody (*8, 15*).

The nonspecific effects of *C. parvum* have been attributed primarily to the stimulation of the reticuloendothelial system after treatment with systemic *C. parvum* (*8*), and the present experiments have demonstrated that *C. parvum*-activated macrophages are indeed cytostatic for tumor cells in vitro. A recent report confirms this activity of *C. parvum*-stimulated peritoneal macrophages against leukemia and fibrosarcoma cells in vitro and also shows alveolar macrophages to be similarly activated after iv administration of *C. parvum* (*16*). Nonspecific in vitro inhibition and, in some cases, killing of tumor cells by glass-adherent peritoneal cells has also been described after their activation with such nonspecific agents as *Toxoplasma gondii* and BCG and also with endotoxin in similar assay systems (*17–20*). The radioresistance of such cells (*20*) and the lack of antitumor activity in thioglycolate-stimulated macrophages (*17*) have also been documented. The process is reported to be nonspecifically selective for malignant cells and is envisioned as an in vivo surveillance mechanism (*18, 19*); such activated cells, especially since they are not restricted to fixed lymphoid organs, would seem ideally suited for "mopping up" metastases. The ability of animals stimulated systemically with *C. parvum* to deal with blood-borne tumor cells has been demonstrated here. A recent report confirms that systemic *C. granulosum*, which is similar to *C. parvum* in its lymphoreticular stimulatory properties (*7*), greatly reduces the number and size of artificial pulmonary metastases in mice (*21*).

There are various negative considerations for the use of systemic *C. parvum* as a therapeutic antitumor agent for solid tumors. Toxicity problems were encountered with the high doses required for optimal tumor suppression in the mouse system. Another aspect is that cell-mediated immunity is considered essential in the immunorejection of most tumors, but there has been accumulating evidence that systemically administered *C. parvum* results in a nonspecific depression of T-cell activity (*22–25*). An instance of reduced specific tumor immunity after iv treatment with *C. parvum* has been reported (*26*). Recent work shows, however, that depression of delayed-type hypersensitivity to an unrelated antigen occurs only when *C. parvum* is administered iv but not sc—the depression phenomenon is mediated, apparently, by lymphocyte trapping in the *C. parvum*-stimulated spleen (*21*). Finally, no specific antitumor immunity develops as a result of systemic *C. parvum* treatment of solid tumors. Such immunity would be expected to be critical in case of possible "escapes" after only partially successful treatment. In light of these considerations and the overall effectiveness of iv treatment with *C. parvum*, the companion paper considers various aspects of sc *C. parvum* therapy in the same tumor model (*5*).

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Corynebacterium parvum as a Therapeutic Antitumor Agent in Mice. II. Local Injection[1,2]

Martin T. Scott[3,4]

SUMMARY—Intratumor (it) injection of *Corynebacterium parvum* into the solid tumor mastocytoma P815 caused marked inhibition of tumor growth, increased survival time, and 44% complete regressions. The protective effect of it-injected *C. parvum* was entirely T-cell-dependent and "cured" mice were specifically immune to rechallenge. Specific pre-emption of the peripheral immune response to *C. parvum* reduced the effectiveness of it injections of *C. parvum*; thus its antitumor activity was mediated in part by nonspecific "fallout" from the specific immune response (probably delayed-type hypersensitivity) to *C. parvum*. *C. parvum* injected subcutaneously (sc) at a site distant from the tumor only minimally affected tumor growth, but *C. parvum* stimulation of the tumor-draining node, though less effective than it injection, markedly inhibited tumor growth. The relative merits of sc and intravenous *C. parvum* therapy were discussed.—J Natl Cancer Inst 53: 861–865, 1974.

THE PRECEDING PAPER described the use of systemically administered *Corynebacterium parvum* as a therapeutic antitumor agent in mice and showed it to be largely nonspecific in its mode of action (*1*). This report deals similarly with the subcutaneous (sc) administration of *C. parvum*, primarily into the tumor site, with the same tumor model. *C. parvum* injected sc causes marked stimulation of the draining lymph node, as evidenced by increase in both mass and DNA synthesis (*2*). Resulting systemic stimulation, judged by hepatomegaly and splenomegaly, is only marginal.

MATERIALS AND METHODS

The tumor system.—The characteristics of the mastocytoma P815 (MA) growing in the foot pads of (C57BL/6 X DBA/2)F_1 (B6D2F_1) mice and the method of measurement were detailed in (*1*). Complete regressions were recorded when tumors grew to a footpad thickness of at least 3 mm and then regressed completely. Only mice remaining tumor free for at least 100 days were used for rechallenge experiments.

C. parvum.—Batch #PX289 from Wellcome Research Laboratories, Beckenham, Kent, England, was used.

Sheep red blood cells (SRBC).—SRBC in Alsever's solution were purchased from the Animal Blood Center, Syracuse, New York. They were washed 3 times in saline before use.

T-cell-deprived mice.—B6D2F_1 mice were prepared as described in (*1*).

DNA assay.—DNA synthesis in the popliteal lymph node after injection of material into the foot pad was assessed by injecting 20 μCi tritiated thymidine (New England Nuclear Corp., Boston, Mass.) intravenously (iv) 30 minutes before the node was removed. The DNA/node was assayed by trichloroacetic acid extraction and liquid scintillation counting as described in (*3*).

RESULTS

Effect of Intratumor (it) *C. parvum* Injection

Six days after sc injection of 10^6 live MA cells into a hind foot pad, mice received various amounts of *C. parvum* in 0.05 ml saline sc into the same site. Injection of *C. parvum* into normal foot pads caused some swelling within 24 hours; this was directly proportional to the *C. parvum* dose. The swelling always subsided without lasting effect. Thus in the experimental groups shown in text-figure 1, the initial rapid increase in foot-pad thickness after high doses of *C. parvum* is attributable to this nonspecific swelling and not to a rapid increase in tumor growth. All concentrations of *C. parvum* injected into the tumor lesion inhibited tumor growth rate, and some complete regressions occurred with all but the lowest *C. parvum* dose (7 μg). The optimal dose was 70 μg, with 4 of 6 complete regressions in this experiment. That 350 μg was less effective than 70 μg has been borne out in repeated experiments; the total cure rates to date were 20 and 44%, respectively. Injection of *C. parvum* it seemed highly effective, for complete regressions were still achieved by treatment given as late as 12 days after tumor establishment, when tumor-bearing feet were twice their normal thickness (text-fig. 2). As expected, the smaller the tumor mass, the more effective the therapy.

Location of sc Injection of *C. parvum*

The question arose as to whether the *C. parvum* needed to be injected directly into the tumor site to effect its antitumor activity. Mice that had had 10^6 live MA cells established in the right hind foot pads 6 days earlier received 70 μg *C. parvum* sc at various sites in relation to the tumor (text-fig. 3). *C. parvum* injected into the contralateral foot pad had a minimal effect on the growth rate of the tumor and only slightly increased the overall survival time of the animals. *C. parvum* injected below the knee in the right hind leg (i.e., stimulating the tumor-draining node but not injected directly into the tumor site) caused inhibition of tumor growth rate and prolonged survival times; however, no complete regres-

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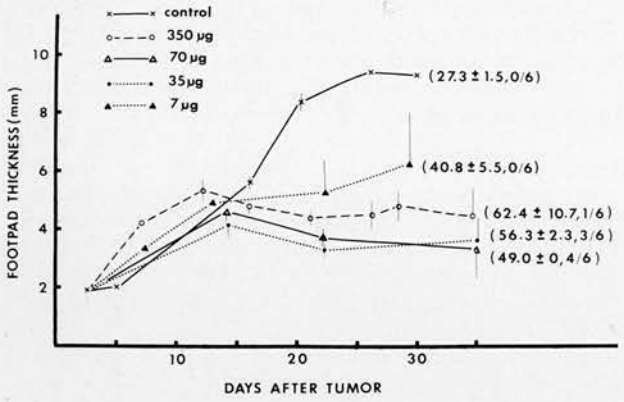
[3] Trudeau Institute, Inc., P.O. Box 59, Saranac Lake, N.Y. 12983.

[4] The excellent technical assistance of Sandra L. Warner is gratefully acknowledged.

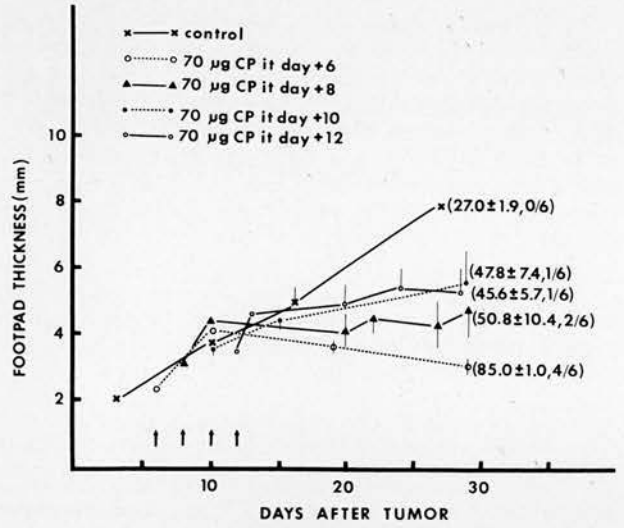
sions occurred. Injection into the tumor site caused the maximum inhibition of tumor growth with the usual high proportion of complete regressions.

Effect of it Injection of *C. parvum* in T-Cell-Deprived Mice

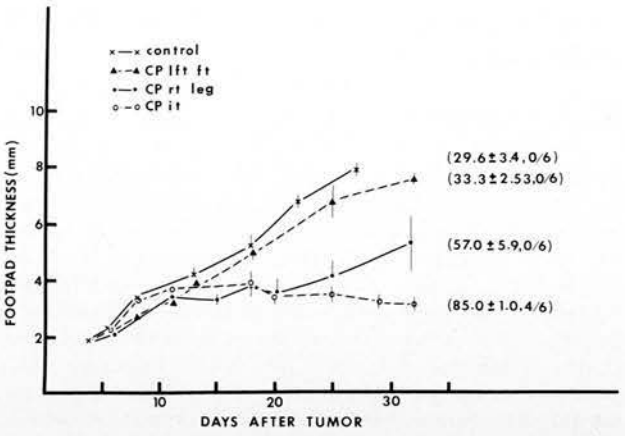
The kinetics of growth of the MA in T-cell-deprived mice were similar to those in intact mice, but overall survival time was markedly reduced. *C. parvum* injected into the tumor site 6 days after tumor establishment in T-cell-deprived mice caused no inhibition of tumor growth rate and no increase in survival time (text-fig. 4). The nonspecific swelling following *C. parvum* injection was evident, however, in such mice; apparently this local effect of *C. parvum* had no antitumor effect.



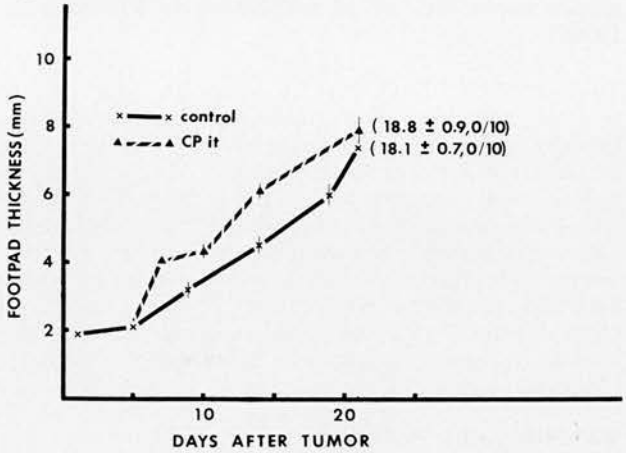
TEXT-FIGURE 1.—Effect of various doses of *C. parvum* injected sc into the tumor site, 6 days after tumor establishment. Numbers in *parentheses* represent mean survival time ± SE of those animals that died, and number of survivors/No. in experimental group.



TEXT-FIGURE 2.—Effect of it injection of *C. parvum* (CP) on tumors of increasing size. At either 6, 8, 10, or 12 days after injection of 10^6 live MA cells into a hind foot pad, mice received 70 µg CP in the tumor site. *Numbers in parentheses* as in text-figure 1.



TEXT-FIGURE 3.—Effect of sc injection of CP at various sites in relation to an ongoing tumor. Six days after injection of 10^6 MA cells into the right hind foot pad, mice received 70 µg CP in the contralateral foot (▲▰▰▲▲), in the right leg below the knee (●▬▬●), or directly it (●▰▰●). Control mice received no treatment (x▬▬x). *Numbers in parentheses* as in text-figure 1.



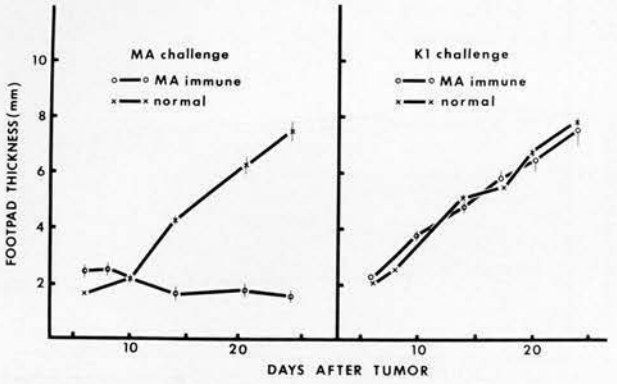
TEXT-FIGURE 4.—Effect of it injection of CP (350 µg) 6 days after establishment of tumor in T-cell-deprived mice. *Numbers in parentheses* as in text-figure 1.

Immune Status of Cured Animals

To test for systemic antitumor immunity in animals whose tumors had completely regressed after it injections of *C. parvum*, these animals were rechallenged with 10^6 live MA cells in the foot pads contralateral to the original tumor. Another group of cured animals was challenged with 10^6 cells from an unrelated fibrosarcoma (K1) induced in $B6D2F_1$ mice by benzpyrene (text-fig. 5). Immunity to the MA was evidenced by the complete disappearance of the tumor after a short period of initial growth. Growth of K1 in normal and MA-immune mice was similar, showing the specific nature of the immunity.

Delayed-Type Hypersensitivity (DTH) to *C. parvum*

The attribute of nonspecifically stimulating lymphoid and macrophage elements with a resultant enhancement of resistance to a wide range of tumors is shared by *C. parvum* and BCG. Suppression of tumor



TEXT-FIGURE 5.—Mice whose foot-pad tumors (MA) had undergone complete regression following it injections of *C. parvum* (MA immune) were challenged with either 10^6 live MA cells or 10^6 live K1 fibrosarcoma cells in the contralateral foot pads, and the growth of those tumors was monitored. Control mice (normal) had no previous experience of either tumor.

growth at a site of infection with living BCG is mediated by the host during a DTH response to the infecting organisms (*4*, *5*), and it thus seemed that the antitumor activity of it injections of *C. parvum* might be similarly effected. Experiments were undertaken to see if delayed-type reactivity develops toward *C. parvum*.

Mice were sensitized with various doses of *C. parvum* injected sc into a hind foot pad and, at the intervals shown, received an eliciting dose of *C. parvum* into the contralateral foot pad. The thickness of the latter foot pad was measured with a Schnelltaster dial gauge caliper 3 hours (Arthus reactivity) and 24 hours (delayed reactivity) later. Each day, control groups of mice received only the eliciting dose of *C. parvum* to control for nonspecific swelling; the resultant figures were subtracted from those of the experimental groups to give the increase in foot-pad thickness attributable to *C. parvum* sensitization. All doses of *C. parvum* caused delayed reactivity, which peaked 6 days after sensitization; none of the doses caused any significant immediate reactivity (text-fig. 6). The experiments were repeated with very low eliciting doses of *C. parvum* (3.5 μg) that caused no nonspecific swelling, and the same results were obtained. Further experiments showed that neither 700 μg nor 350 μg *C. parvum* injected iv resulted in any detectable delayed reactivity. This accords with the recent report that DTH sensitization with particulate antigens is more readily achieved with sc than iv injection (*6*).

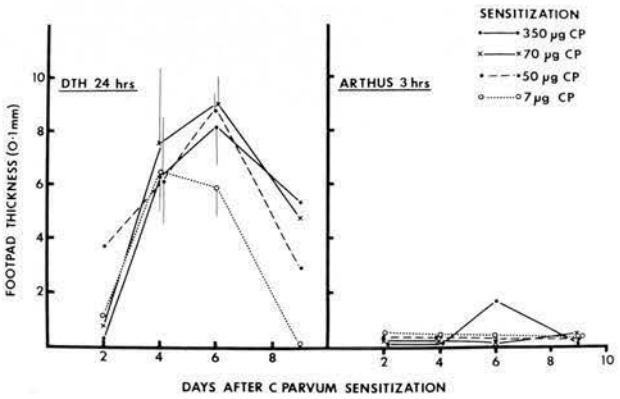
Pre-emption of Local Immune Response to *C. parvum*

To determine the function of the local development of immunity to *C. parvum*, advantage was taken of the phenomenon known as "pre-emption," in which the capacity to mount a peripheral immune response to an antigen is abolished by previous systemic injection of the antigen (*7*). The mechanism underlying this phenomenon is not known, but it is most probably based on the principle that the immune response to the pre-empting antigen takes place largely in the spleen, and subsequent failure of the peripheral lymph node response results from sequestration in the spleen of those cells capable of responding to the antigen (*7*).

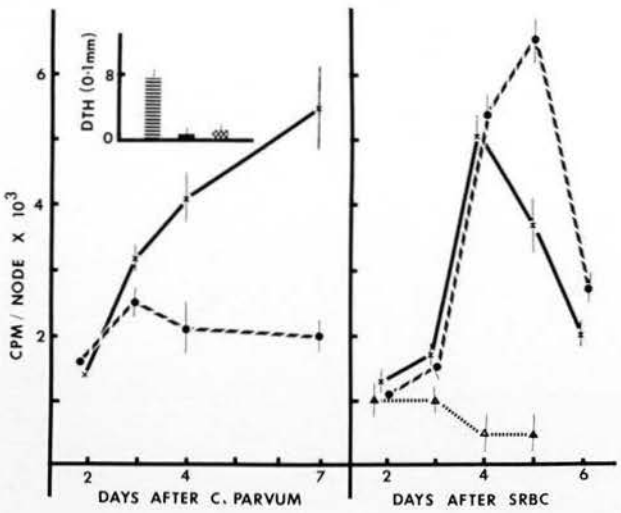
In these experiments, pre-emption was achieved by a single iv injection of *C. parvum* (700 μg) 4 days before a sensitizing foot-pad injection of *C. parvum* (350 μg). DNA synthesis in the draining popliteal node was compared in normal and pre-empted animals and found to be almost totally abolished in pre-empted animals (text-fig. 7, *left*). DTH to *C. parvum*, which normally arises from foot-pad sensitization with *C. parvum* (text-fig. 6), was also abolished by the previous iv injection of *C. parvum* (text-fig. 7, *left, inset*). The specificity of the pre-emption is demonstrated in text-figure 7, *right*. Mice that received *C. parvum* iv showed an unimpaired node-proliferative response to SRBC, an unrelated antigen, whereas this was abolished by earlier iv injection of a large dose of SRBC.

Function of Immune Response to *C. parvum* in the Antitumor Effect of it-Injected *C. parvum*

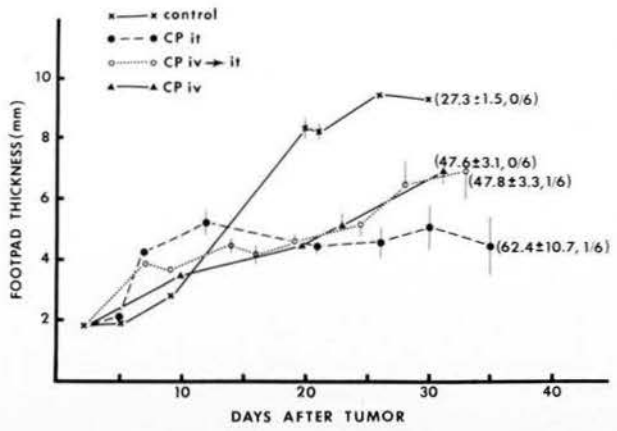
To determine whether the immune response to *C. parvum* was a component of the antitumor effect of it-injected *C. parvum*, experiments were designed to test whether its effect would be modified in animals that were pre-empted to *C. parvum* (i.e., unable to develop a peripheral immune response to *C. pervum*). Live MA cells were injected into a hind foot pad, and 2 days later the mice received the pre-empting dose of *C. parvum* iv; 4 days later, *C. parvum* was injected into the tumor site and the tumor was monitored for



TEXT-FIGURE 6.—DTH and Arthus reactivity to CP. Mice received various sensitizing doses of CP sc into a hind foot pad. At the times shown, an eliciting dose of CP (350 μg) was injected sc into the contralateral foot pad and the foot-pad thickness was measured at 3 and 24 hours. For each time point, a group of normal mice received the eliciting injection of CP. Graph shows the difference in foot-pad thickness between normal and experimental groups.



TEXT-FIGURE 7.—Specific ablation of the peripheral immune response to *C. parvum* by prior systemic injection of *C. parvum*. *Left:* Rate of DNA synthesis (incorporation of tritiated thymidine into DNA) in popliteal lymph nodes after foot pad was inoculated with 3.5 μg *C. parvum* in normal mice (x▬x) or mice that had recieved *C. parvum* (700 μg) iv 4 days earlier (●▬●). *Inset:* 24-hour foot-pad swelling (DTH reactivity) in response to a foot-pad injection of 350 μg *C. parvum* in normal mice (*crossed bars*), in mice that had been sensitized with 350 μg *C. parvum* sc in the contralateral foot pad 6 days before (*horizontal bars*), or in mice that received *C. parvum* (700 μg) i v 4 days prior to such sensitization (*solid*). *Right:* DNA synthesis in popliteal lymph nodes in response to a foot pad injection of 10^8 SRBC in normal mice (x▬x) or in mice that had received either 700 μg *C. parvum* iv (●▬●) or 10^9 SRBC iv (▲◤◢▲) 4 days earlier. *Vertical bars* represent ± SE (n=5).



TEXT-FIGURE 8.—Effect of CP on inhibition of tumor growth by it injection of CP. Mice received either CP (350 μg) injected it 6 days after tumor establishment (●▬●), CP (700 μg) iv 2 days after tumor (▲◤◢▲), or CP both iv and it at 2 and 6 days after tumor, respectively (○◤◢○). Control animals received no treatment. Numbers in parentheses as in text-figure 1.

subsequent growth compared with all the requisite controls (text-fig. 8). *C. parvum* given iv alone produced its usual transitory inhibition of tumor growth and increase in survival time (*1*). *C. parvum* injected into the tumor caused marked inhibition of tumor growth and some complete regressions. Injection of *C. parvum* iv prior to it injection of *C. parvum* reduced the effect of the it injection. The growth rate of the tumor was similar to that seen after iv administration of *C. parvum* alone, as was the mean survival time of animals that died. The reduction in effect of it-injected *C. parvum* by prior iv treatment with *C. parvum* was not significant at the overall survival level; however, the differences in the mean sizes of the progressively growing tumor became significant at day 30 ($P<0.025$). The experiment was repeated with similar results: it-injected *C. parvum* alone resulted in a survival time of 49.2±6 days (mean ± SE) with 2 cures. The pre-empted group's survival time was 45.0±2 days with 1 cure, and the difference in tumor size between these groups was significant from 20 days. That some regressions did occur in the pre-empted groups is significant, since *C. parvum* given iv alone 2 days after tumor establishment has never caused any complete regressions (*1*).

DISCUSSION

C. parvum injected directly into the site of an ongoing tumor is highly inhibitory for tumor growth and causes some complete regressions. Optimal results were achieved when the *C. parvum* was injected into the tumor site; however, stimulation of the tumor-draining lymph node by *C. parvum* not injected into the tumor site also markedly inhibited tumor growth, but did not achieve any complete regressions. These findings agree with a recent report describing complete regressions of mouse adenocarcinomas after it *C. parvum* injection (*8*). With both the adenocarcinoma model and this MA, mice whose tumors had regressed completely under the influence of it-injected *C. parvum* exhibited strong specific antitumor immunity upon rechallenge (*8*). This was so despite the fact that the MA is extremely poorly immunogenic in $B6D2F_1$ mice (*1*).

The antitumor effect of it-injected *C. parvum* is not attributable to any intrinsic antitumor activity of the *C. parvum* itself, but requires mediation by the host's own immune system as evidenced by the abrogation of the effect in T-cell-deprived mice.

The optimal it dose of *C. parvum* was 70 μg; 350 μg was consistently less effective. This dose discrepancy may be due to overstimulation of the draining node by the higher dose of *C. parvum*. Destructive changes in regional lymph nodes after foot-pad injection of highly virulent strains of BCG have been reported (*9*).

Pre-emption of the peripheral immune response to *C. parvum* shows that a component of the mode of antitumor action of it-injected *C. parvum* results from the development of the immune response to *C. parvum* itself. That the development of DTH reactivity to BCG is responsible for its antitumor effect (*4*, *5*), along with the demonstration of the T-cell-dependent nature of *C. parvum* reaction, suggests that the DTH component of the immune response to *C. parvum* may also be responsible. This is, however, equivocal, since pre-emption of the peripheral immune response to *C. parvum* abolished all aspects of that response as measured by cell proliferation. Other studies have also described tumor regression at the site of DTH

reactions to skin-sensitizing agents (*10*). Since stimulation of the tumor-draining node by *C. parvum* not injected into the tumor site caused marked inhibition of tumor growth, interaction between *C. parvum* and tumor cells may also occur by means of the regional lymphatics. Not all the antitumor activity of *C. parvum* is attributable to a nonspecific "bystander" effect, since ablation of the peripheral immune response to *C. parvum* did not completely abolish its antitumor effect. The residual activity is readily attributable to the intrinsic nonspecific antitumor activity of *C. parvum*-activated macrophages as described in (*1*). Indeed, 4 days after an it injection of *C. parvum*, histologic studies have shown large numbers of macrophages throughout the tumor (*8*). That the antitumor is not totally correlated with DTH reactivity to *C. parvum* is further evidenced by the finding that, whereas all doses of *C. parvum* (350–7 μg) produced equivalent high levels of DTH (text-fig. 6), the highest and lowest doses were considerably less efficient at destroying the tumor (text-fig. 1).

Injection of *C. parvum* it has thus been shown, in this and other tumor systems, to be highly effective in causing complete regressions of large tumors (*8*). Reference to the previous paper (*1*) allows a comparison of the present antitumor effects with those of systemically administered *C. parvum*. The it injection is considerably more effective than iv injection in inhibiting tumor growth; *C. parvum* injected iv only 2 days after tumor establishment (compared with 6 days for the it injection) caused only transient inhibition of growth and no complete regressions. The it injection has the added advantage of producing systemic specific antitumor immunity which *C. parvum* injected iv failed to do. *C. parvum* injected sc near, but not into, the tumor site again caused more inhibition than *C. parvum* iv, though both were less effective than the it injection. Likhite and Halpern (*8*) obtained similar results with multiple injections of *C. parvum* used against a mammary adenocarcinoma in mice; sc injections of *C. parvum* near the tumor site were less effective than it injections but more effective than intraperitoneal injections (*8*).

The MA P815 metastasizes but does not readily lend itself to a study of metastases, since the lesions are highly inconsistent. With regard to the effect of sc inoculations of *C. parvum* on blood-borne tumor cells, previous work has demonstrated that *C. parvum* injected sc into the neck region confers no protection against a subsequent low-dose MA challenge iv, whereas *C. parvum* injected iv markedly increased survival times (*1*). Another study of a fibrosarcoma that produces consistent experimental metastases in the lung after iv injection describes, however, that sc injection of *C. parvum* significantly reduced the number of metastases generated on the lung (*11*).

Other considerations favoring local rather than systemic administration of *C. parvum* are as follows: It is effective at relatively low doses, thus eliminating the toxicity problems encountered with the high doses required for the antitumor effects after systemic administration of *C. parvum* (*1*). Also evident is the clinical preference for local rather than systemic administration of any particulate material. Further work has now also shown that local administration of *C. parvum* circumvents the possibility of nonspecific T-cell depression that often accompanies systemic injection of *C. parvum* (*2*). For optimal effects, *C. parvum* given sc should be administered either directly into the tumor or as close as possible to the tumor site. Ongoing studies show that this problem may be alleviated to a certain extent by utilizing the marked systemic immunity that results from an injection of *C. parvum* mixed with irradiated tumor cells (Scott, in preparation).

It appears that the mode of action of it-injected *C. parvum* is similar to that of BCG in that a specific immune response to these agents nonspecifically suppresses tumor cells at the site of reaction (*4*, *5*). The apparent advantage of *C. parvum* over BCG is that it is effective in killed form, thereby circumventing such problems as loss of viability and only retrospective knowledge of actual dose administered. Further, all dangers of resulting systemic infection are eliminated. Recent data indicates that sc injection of viable BCG is not as effective in reducing the incidence of pulmonary metastases in mice as is *C. granulosum* (*12*), which is closely related to *C. parvum* in its lymphoreticular-stimulating properties (*13*). Studies designed to compare the antitumor capabilities of *C. parvum* and BCG against MA P815 are now in progress.

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Potentiation of the Tumor-Specific Immune Response by *Corynebacterium parvum* [1,2]

Martin T. Scott [3,4]

SUMMARY—Strong, specific, cell-mediated antitumor immunity resulted from the sc injection into mice of *Corynebacterium parvum* (CP) mixed with irradiated cells from mastocytoma P815 (MA), whereas injection of irradiated MA alone was without effect. Maximum immunity was achieved with small doses of CP, and the injection sites of CP and irradiated MA did not need to be coincident but required common lymphoid drainage for immunity to result. The process was largely T-cell dependent but not dependent on the development of a specific immune response to CP; it was also demonstrable and effective in a therapy situation. No such immunity was evident after systemic administration of CP and irradiated MA. Simultaneous systemic injection of CP and irradiated MA did, however, nonspecifically reduce the enhancing effect of systemic injection of irradiated MA alone. J Natl Cancer Inst 55: 65–72, 1975.

SOME ANEROBIC CORYNEFORMS with lymphoreticular stimulating properties, notably *Corynebacterium parvum* (CP) and *C. granulosum*, exhibit antitumor activity in animals (*1–8*) and man (*9, 10*) when given in the form of killed vaccines. Interest in these organisms as anticancer agents originally arose because of their marked stimulatory effect on phagocytic cells, and macrophages activated by CP and *C. granulosum* have subsequently been demonstrated to nonspecifically inhibit tumor cell growth in vitro (*7, 11–13*). Evidence has accumulated that the in vivo antitumor effects of CP may be largely nonspecifically mediated, presumably by such an activated macrophage system. The in vivo antitumor activity of CP is operative in T-cell-deprived (*7, 14*) and antilymphocyte serum-treated mice (*15*). It is also radioresistant (*4*) and effective in mice genetically selected for low antibody responses (*16*). Under some circumstances, augmentation of specific antitumor responses may also be a component of CP's mode of antitumor activity as suggested by the recent findings that mice whose tumors had completely regressed after CP was given intralesionally (*3, 8*) were highly and specifically immune to subsequent tumor challenge.

This paper describes the strong, specific, cell-mediated immunity that results from the sc injection of CP mixed with irradiated tumor cells into mice, and the failure to achieve such immunity with systemic injections. The optimal immunizing conditions are described, and the efficacy of such immunity in a therapy model is demonstrated.

MATERIALS AND METHODS

Animals.—Adult male (C57BL/6×DBA/2)F_1 (B6D2F_1) mice were used.

T-cell-deprived mice.—Thymectomized, irradiated, bone-marrow-reconstituted mice were prepared as in (*7*).

CP.—A formal-killed suspension of CP (7 mg/ml) was obtained from Burroughs Wellcome Co., Research Triangle Park, North Carolina. Subsequent dilutions were made with saline.

Tumor.—Mastocytoma P815 (MA) was originally induced in a DBA/2 mouse by 3-methylcholanthrene. The experimental use and characteristics of MA in B6D2F_1 mice were described in (*7, 8*). The tumor was maintained in ascites form by weekly passage, and various numbers of tumor cells in Hanks' balanced salt solution (HBSS) containing 20 U heparin per ml were injected sc either into the footpad (0.05 ml with a 30-gauge needle) or the thigh (0.1 ml with a 26-gauge needle). Tumor growth in the footpad was measured as footpad thickness by a dial gauge caliper (Schnelltaster, H. C. Kroplin GMBH, Hessen, West Germany). Tumor growth in the thigh was measured as two diameters at right angles, the size being expressed as the multiplicand. Once the tumor became necrotic, the measurements were discontinued and the mice scored for survival only. Mean tumor sizes at various time points were compared by the Student's *t*-test, and relevant differences were noted.

Irradiation of tumor cells.—Tumor cells (10^7/ml) to be irradiated were suspended in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (Flow Laboratories, Rockville, Md.). Six thousand rads of γ-irradiation were delivered from a ^{137}Cs source at a dose rate of 32 rads per minute. After irradiation, the cells were resuspended in HBSS (20 U heparin/ml) before injection.

RESULTS

Tumor Immunity Resulting From Local Injection of CP-Irradiated Tumor Cell Mixtures

Mice were given a mixture of 10^7 irradiated MA cells and 175 μg CP sc in the left hind footpad. They were challenged 7 days later with 10^6 live MA cells injected into the contralateral footpad, and the growth of this tumor was monitored. Control groups were either untreated or pretreated with irradiated MA or CP alone (text-fig. 1). The growth of 10^6 MA cells in the footpads of normal mice conformed to that described previously for this tumor model and no survivors were recorded (*7, 8*). Injection of CP alone did not influence tumor growth as evidenced by tumor size and mean survival data. Injection of irradiated MA alone also did not significantly modify tumor growth. The mean survival time of these animals was marginally less than for normal mice ($P<0.1$) in the experiment shown; however, repeat experiments showed no overall statistically significant difference in survival time for these two groups. Injection of CP-irradiated MA mixtures markedly inhibited tumor growth (in some cases, completely) and increased survival times. The systemic na-

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[4] The excellent technical assistance of Sandra L. Warner is gratefully acknowledged.

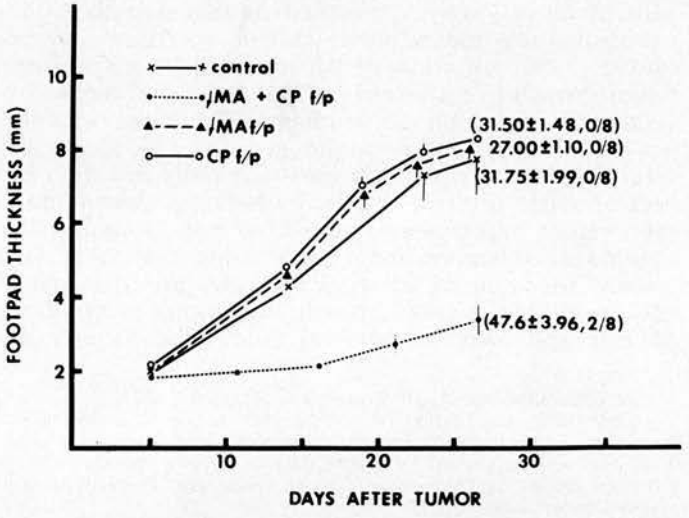
ture of the tumor immunity after injection of CP-irradiated MA mixtures was further demonstrated in experiments where the challenge MA cells (10^4) were injected iv and survival data recorded. Normal mice all succumbed to the iv challenge, with a mean survival time of 20.5 ± 1.6 (SE) days, whereas only 3 of the 10 mice pretreated with CP-irradiated MA mixtures died during a 60-day period.

Dose of CP in the Mixtures

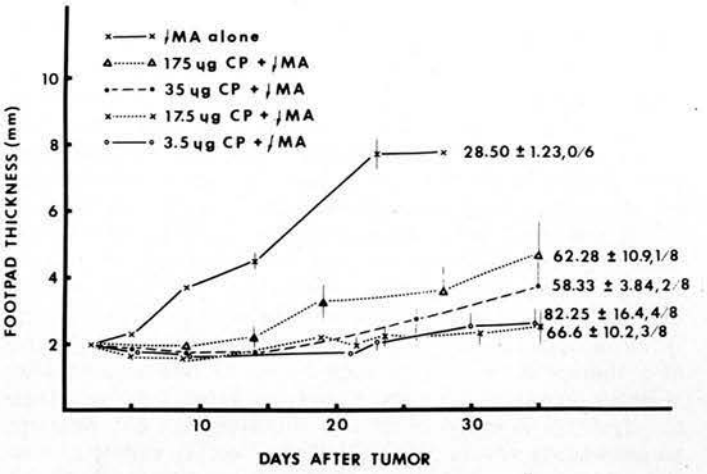
The effect of varying the amount of CP in the CP-irradiated MA mixtures was investigated. Groups of mice received 10^7 irradiated MA cells mixed with different amounts of CP sc into the hind footpad. Tumor was challenged 7 days later in the contralateral footpad (text-fig. 2). The lowest dose of CP (3.5 μg) was most effective at producing tumor immunity; it caused four complete inhibitions of tumor compared with only one in mice given 175 μg CP. The difference in tumor size between these two groups was also significant ($P < 0.05$) from day 14 onward. Accumulated results from repeated experiments showed cure rates for 175-μg and 3.5-μg groups of 20.0 ± 3.1 (SE) and $45.8 \pm 4\%$, respectively. There was no significant difference in tumor size between the 3.5-μg and the 17.5-μg groups throughout the experiment, and the 35-μg group appeared intermediate between the 175-μg and lower-dose group, as judged by tumor size and number of complete inhibitions.

Dose of Irradiated MA Cells in Mixtures

Between $1\text{–}2 \times 10^7$ irradiated MA cells was the highest number practical to include in the mixtures because of the small injection volumes required for footpad inoculation. Less than this number of irradiated MA cells (10^6, 10^5, 10^4) mixed with CP gave significantly less immunity to the tumor challenge, as judged by tumor growth and overall mean survival times.



TEXT-FIGURE 1.—Tumor immunity arising from sc injection of CP-irradiated MA mixtures. Mice were inoculated sc in the left hind footpad with 175 μg CP (○——○), 10^7 irradiated MA (▲— —▲), or a mixture of 10^7 irradiated MA and 175 μg CP (●- - -●). Controls were untreated (×——×). Seven days later, all mice received 10^6 live MA cells in the contralateral footpad and growth of this tumor was measured. *Vertical bars* represent ±SE. *Numbers* are: mean survival time ±SE of those mice that died, number of survivors/number in experimental group.



TEXT-FIGURE 2.—Effect of different doses of CP in CP-irradiated MA mixtures. Various doses of CP were mixed with 10^7 irradiated MA and injected sc into the left hind footpad. Seven days later, 10^6 live MA cells were injected into the contralateral footpad and growth of this tumor was measured. *Numbers* are as in text-figure 1.

Duration and Specificity of Tumor Immunity

Duration of the immunity was ascertained by the rechallenge of mice whose previous challenge tumors had completely regressed. A group of 7 such mice from various experiments were rechallenged between 99 and 160 days after their initial tumor challenge. Transient tumor growth was evident in 5 of these mice but the tumor subsequently regressed, which left all the mice tumor free by day 30. Controls all died with large tumors; their mean survival time was 27.2 days. Specificity of the immunity arising from the injection of CP-irradiated MA mixtures was tested by challenge of MA-immune mice with 10^6 live cells from an antigenically unrelated, benzpyrene-induced fibrosarcoma K1 (*8*). Growth of the fibrosarcoma in the footpad of MA-immune mice was no different from that in normal mice.

Effect of Varying the Site of Injection of Irradiated MA Cells and CP

To determine whether CP and tumor antigen need to interact at the same site to produce tumor immunity, the following experiment was designed to effect differing degrees of lymph node mediation between the MA cells and CP. All mice received irradiated MA cells sc in the left hind footpad. In the first group, the irradiated MA cells were mixed with CP; other groups received CP as follows: sc below the knee of the left leg, sc into the thigh of the left leg, or sc into the neck region. Controls received only irradiated MA in the left footpad. Seven days later, all groups were challenged with live MA in the right hind footpad and the growth of this tumor was monitored (text-fig. 3). The combination of CP into the neck and irradiated MA in the footpad caused no tumor immunity as evidenced by the lack of influence on the challenge tumor. CP injected into the thigh was expected to involve the popliteal node draining the site of immunization with MA (at least to some extent). CP at this site did significantly inhibit tumor growth and prolong survival time; but inhibition of tumor growth was less than that seen after injection of CP and irradiated MA into the same site. The difference in tumor size between these groups was significant

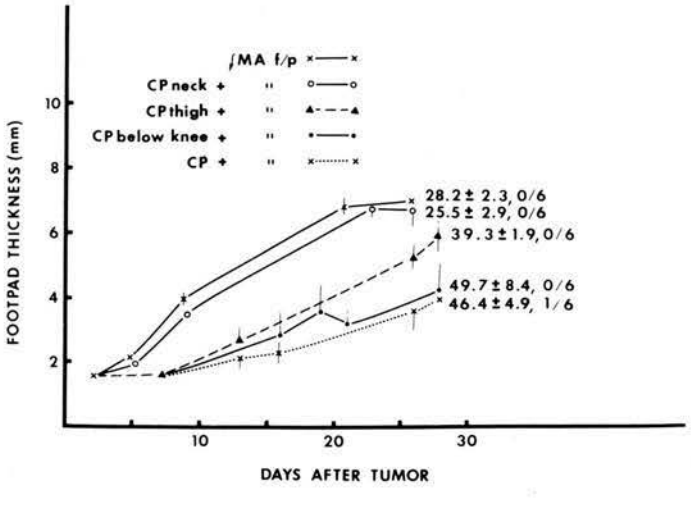
($P<0.05$) from day 19 onward. CP injected into the leg below the knee would be likely to fully engage the node receiving the stimulus from the irradiated MA and, as expected, inhibition of tumor growth in this instance was not significantly different from that in mice receiving CP mixed with irradiated MA in the footpad. In the latter group, however, there was one instance of complete tumor inhibition.

Lymph Node Cell Transfer Studies

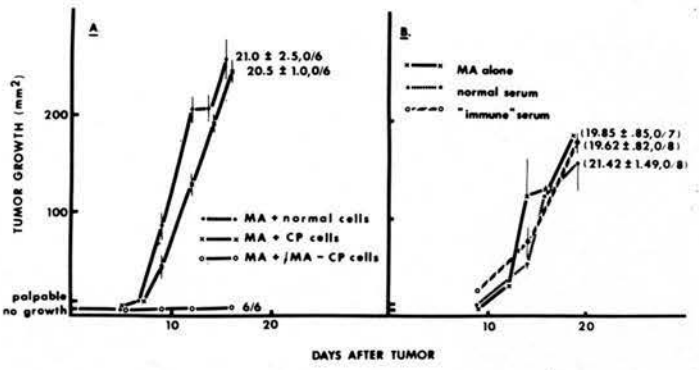
To determine whether the CP-induced tumor immunity was cell mediated, lymph node cell suspensions were prepared from the popliteal lymph nodes of mice that had received footpad injections of CP-irradiated MA mixtures 8 days previously. The lymph node cells were mixed with live MA cells at a ratio of 200:1 (2×10^7 lymph node cells:10^5 live MA cells per injection) and injected sc into the thighs of mice which had been sublethally irradiated (400 rads) 24 hours before. Two controls were the growth of MA cells mixed with normal lymph node cells (mesenteric) or cells from popliteal lymph nodes which had been stimulated by CP injection alone (text-fig. 4A). Lymph node cells stimulated with CP-irradiated MA mixtures totally inhibited tumor cell growth, the experiment being terminated at 54 days. Except for the transient inhibition of tumor growth seen around day 12 with lymph node cells stimulated by CP alone, there was no overall difference between the tumor growth and mean survival times of the groups receiving MA mixed with either CP-stimulated cells or normal cells.

Serum Transfer Studies

Serum transfer experiments were done to determine whether the CP-induced tumor immunity was entirely cell mediated. Serum was prepared from mice given CP-irradiated MA mixtures in both hind footpads 10 days before. Normal mice were bled for control serum. Mice sublethally irradiated (400 rads) 24 hours previously received 0.4 ml undiluted serum iv followed by 10^5 live



TEXT-FIGURE 3.—Effect of CP and irradiated MA cells injected at different sites. All mice received 10^7 irradiated MA sc in the left hind footpad. CP (175 μg) was then injected sc into the same site (×– – –×), below the knee of the left leg (●——●), in the left thigh (▲——▲), or into the neck (○——○). Controls received only irradiated MA (×——×). Seven days later all groups were challenged with 10^6 live MA in the right hind footpad and growth of this tumor was measured. *Numbers* are as in text-figure 1.



TEXT-FIGURE 4A.—Transfer of tumor immunity by lymph node cells. Mesenteric lymph node cells (10^7) from normal mice (●——●), or popliteal node cells from mice given either 175 μg CP plus 10^7 irradiated MA (○——○) or only 175 μg CP (×——×) sc into both hind footpads 7 days before were mixed with 10^5 live MA cells and injected sc into the thighs of sublethally irradiated recipients. Growth of the tumor in the thigh was measured. *Numbers* are as in text-figure 1. B. Serum transfers. Live MA cells (10^5) were injected into the thighs of sublethally irradiated recipient mice. Immediately after tumor injection, the mice received 0.4 ml undiluted serum iv from mice either inoculated in both hind footpads with 175 μg CP plus 10^7 irradiated MA (○——○) 10 days before or untreated (●– – –●). Controls received no serum (×——×). *Numbers* are as in text-figure 1.

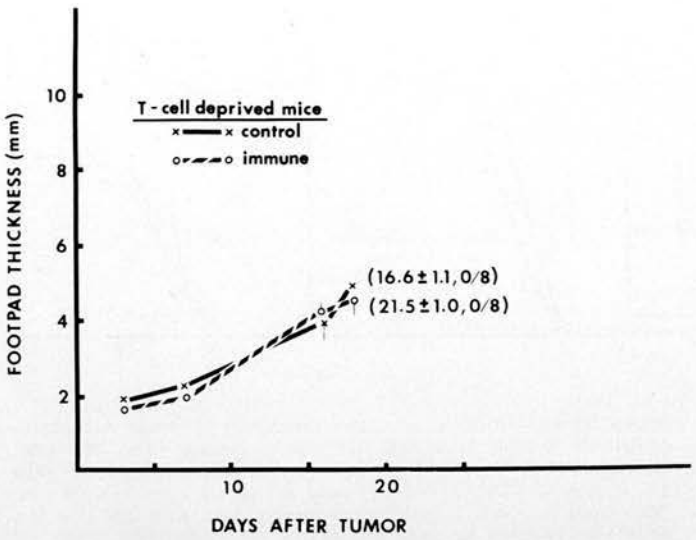
MA cells sc into the thigh. Growth of the tumor in the thigh is shown in text-figure 4B. Both normal and "immune" sera were without effect. A modification of these experiments was to mix the live MA cells with undiluted serum and inject the mixture sc into the thigh; here again no serum-mediated antitumor immunity or enhanced tumor growth was found.

T-cell Dependence of Tumor Immunity

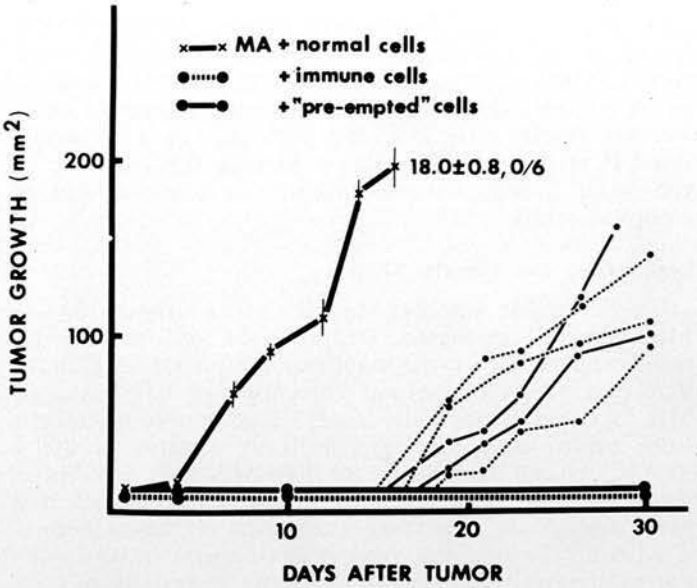
The apparent noninvolvement of serum, along with the ability of the author to transfer the effect with cells, indicated cell-mediated immunity. This prompted an investigation into the T-cell dependence of the phenomenon. T-cell-deprived mice were given CP-irradiated MA cell mixtures sc into the hind footpad, and 7 days later, challenged with 10^6 live MA in the contralateral footpad. Control T-cell-deprived mice were untreated (text-fig. 5). The kinetics of growth of MA in deprived mice was like that in intact mice, but survival times were considerably shorter (*7, 8*). The survival of deprived mice pretreated with the mixture was prolonged ($P<0.01$) compared with untreated deprived mice, but the usual marked inhibition of growth of the solid tumor seen in intact mice (text-figs. 1, 2) was absent; there was no significant difference in tumor size between the groups at any point.

Role of Immune Response to CP in Development of Tumor Immunity

A previous study with $B6D2F_1$ mice detailed how an iv injection of a large dose (700 μg) of CP 4 days before a footpad inoculation of CP makes the popliteal (draining) lymph node specifically unable to proliferate in response to the footpad CP injection (*8*). With the use of this technique, the specific immune response to CP was demonstrated to underlie part of the antitumor activity of an intratumor injection of CP (*8*). This device of "preempting" the local immune response was used here to determine whether the immune response to CP was operationally involved in the generation of spe-



TEXT-FIGURE 5.—Tumor immunity in T-cell-deprived mice. T-cell-deprived mice were inoculated in the left hind footpad with 10^7 irradiated MA plus 175 μg CP (○— —○) or untreated (×——×). Seven days later they were challenged with 10^6 live MA cells in the contralateral footpad and growth of this tumor was measured. *Numbers* are as for text-figure 1.



TEXT-FIGURE 6.—Mesenteric lymph node cells (10^6) from normal mice (×——×), 10^6 popliteal node cells from mice given either a mixture of 3.5 μg CP and 10^7 irradiated MA sc in both hind footpads 8 days previously (●– – –●), or, in addition, a pre-empting dose of 700 μg CP iv 4 days before the mixture (●——●) were mixed with 10^5 live MA cells and injected sc into the thighs of sublethally irradiated recipients. Growth of the tumor in the thigh was measured. *Thin lines* represent tumor growth in individual animals (*see* text). *Numbers* are as in text-figure 1.

cific antitumor immunity under the present conditions. The failure of the preempted nodes to respond to CP in the following experiments was evident from their failure to grow after footpad inoculation compared with normally responding nodes.

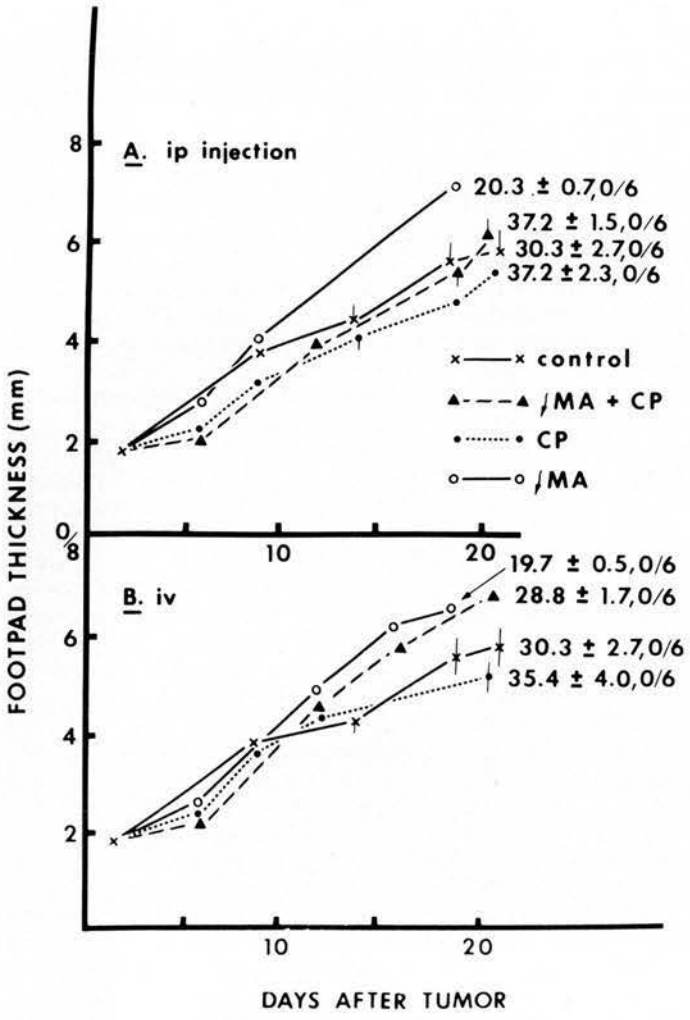
A group of mice received an iv injection of 700 μg CP and, 4 days later, an immunizing dose of CP-irradiated MA mixture into both hind footpads. Controls were untreated or received only the immunizing mixture. Eight days later lymph node cell suspensions were prepared from the popliteal nodes of immunized mice and mesenteric nodes of normal mice. Lymph node cells were mixed with live MA cells at ratios of 50:1, 20:1, and 10:1 and injected into the thighs of mice given 400 rads 1 day before. Each mouse received 10^5 live MA cells. The results of the 10:1 transfer are in text-figure 6. Growth of MA cells mixed with normal cells was evident at 7 days and all mice were dead within 20 days. No tumor growth was obvious until day 14 in the groups receiving either immune cells or preempted-immune cells, and then only 2 of the preempted group and 3 of the unpreempted group developed tumors. A similar relationship was found with cell ratios of 50:1 and 20:1: Only 1 mouse in each group given unpreempted immune cells developed tumors and none appeared in mice receiving preempted cells. The fact that CP-irradiated MA mixtures were fully capable of generating tumor immunity in lymph nodes that had been preempted against responding to CP suggests that immunity to CP was not involved in its mediation of the tumor immune response.

Tumor Immunity Arising From Systemic Injection of CP-Irradiated MA Mixtures

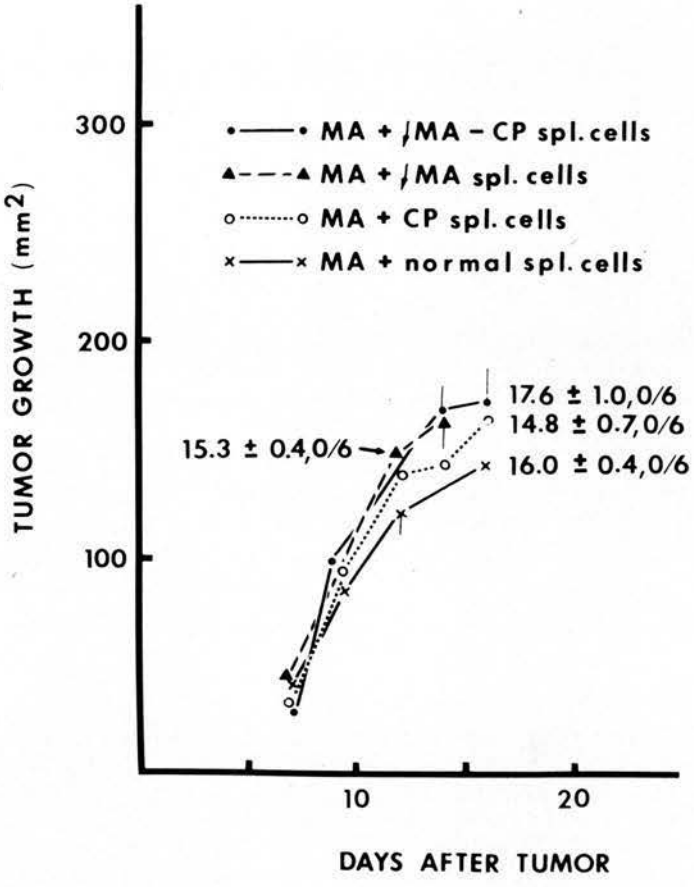
Various considerations, outlined in the "Discussion," led to the speculation that tumor immunity may not result from CP-irradiated MA mixtures injected systemically. To test this, mice were inoculated ip with 5×10^7 irradiated MA cells mixed with 350 μg CP. Control groups received either no treatment, CP, or irradiated MA alone. Seven days later, all mice were challenged in a hind footpad with 10^6 live MA cells and the growth of this tumor was monitored (text-fig. 7). CP alone was marginally but not significantly inhibitory. Injections ip of irradiated MA cells alone enhanced growth of the challenged tumor. This increase in tumor size was highly significant ($P<0.001$) at day 16, as was the reduced overall mean survival time ($P<0.005$). Although the injection of CP-irradiated MA mixtures was no more inhibitory than CP alone, the presence of CP completely abolished the enhancing effect of the irradiated cells. The experiment was repeated by the iv injection route (text-fig. 7B). The dose of irradiated MA cells was reduced to 10^7, since higher numbers caused some immediate deaths due to cell clumping in the inoculum. Again, CP alone did not significantly inhibit tumor growth, and irradiated MA cells alone caused significant tumor enhancement; the difference in tumor size at day 16 and the overall mean survival times between normal and enhanced animals were $P<0.005$ and $P<0.001$, respectively. Injection of CP-irradiated MA mixtures again significantly abolished the enhancing effect of the irradiated MA cells but was no more inhibitory than CP alone. A similar protocol was used in a further experiment where the effect of varying the dose of CP in the mixtures was studied. Irradiated MA cells (10^7) were mixed with either 700, 350, 70, or 7 μg CP. At no dose of CP was the tumor inhibition caused by the mixtures greater than that resulting from CP injection alone.

Spleen Cell Transfers

Systemic injection of CP-irradiated MA mixtures thus failed to confer any overall protection against subsequent tumor challenge, which suggested no development of tumor immunity. A possibility remained, however, that the significant abrogation of the enhancing action



TEXT-FIGURE 7A.—Mice received an ip injection of 5×10^7 irradiated MA plus 350 μg CP (▲——▲), irradiated MA (○——○), CP (●– – –●) alone, or no treatment (×——×). Seven days later they were challenged with 10^6 live MA in a hind footpad and the growth of this tumor was measured. *Numbers* are as in text-figure 1. B. Groups are the same as in A, except pretreatment was by the iv injection route and the irradiated MA dose was reduced to 10^7.

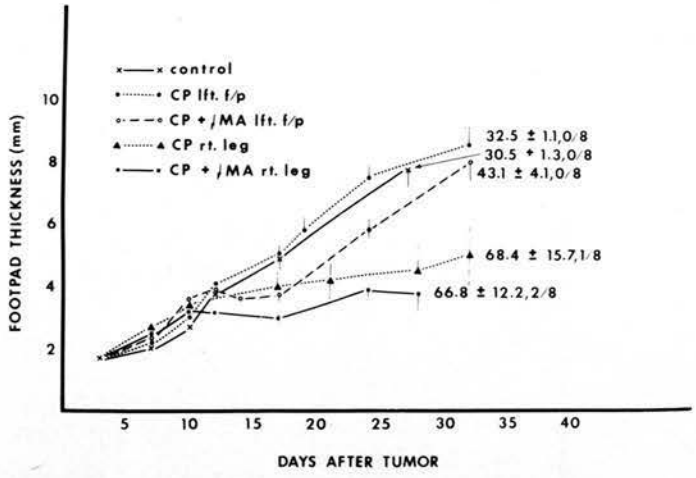


TEXT-FIGURE 8.—Spleen cells, 2×10^7, from normal mice (×——×), mice given 10 irradiated MA cells plus 350 μg CP iv (●——●), irradiated MA (▲——▲), or CP (○– – –○) alone 7 days previously were mixed with 10 live MA cells and injected sc into the thighs of sublethally irradiated mice. Growth of the tumor in the thigh was then measured. *Numbers* are as in text-figure 1.

of irradiated MA alone indicated the development of tumor immunity. To detect any such immunity, spleen cell transfer experiments were performed. Mice were inoculated iv with either CP-irradiated MA mixtures, CP, or irradiated MA cells alone as above. Seven days later spleens were removed and single cell suspensions prepared. The spleen cells were mixed with live MA cells at a ratio of 200:1 (2×10^7 spleen cells:10^5 MA cells per injection) and injected sc into the thighs of mice that had received 400 rads 24 hours before. Growth of the tumor in the thigh was followed for signs of immunity (text-fig. 8). Tumor growth was not inhibited with any spleen cell group, nor were any differences between the groups statistically significant.

Therapeutic Use of CP-Irradiated MA Mixtures

Tumors were established in the right hind footpads of mice by sc injection of 10^6 live MA cells. Three days later, CP-irradiated MA mixtures were injected sc either into the contralateral footpad or below the knee on the tumor-bearing leg. Controls received injections of CP alone at the same sites. Text-figure 9 shows the results of the experiment with a CP dose of 175 μg with 10^7 irradiated MA cells. CP alone in the contralateral footpad was without effect; however, tumor resistance after CP-irradiated MA mixtures was evident from the plateau in tumor growth between days 12 and 17. Following this transient inhibition, tumor growth continued at its normal rate. The increased mean survival time of this group compared to mice treated with CP alone was significant ($P<.05$). Injection of CP alone into a site near the tumor caused marked inhibition of growth, increased survival times, and one complete regression. Tumor growth after injection of CP-irradiated MA mixture at this site was not significantly different from that following CP injection alone at any point in the growth curve, nor were the overall mean survival times of those animals that died. There were, however, two complete regressions in the instance of the mixture injection. Since from the prevention studies (text-fig. 2) it might be predicted that stronger immunity would result if a lower dose of CP were used, the experiment was repeated with 3.5 μg CP in the CP-irradiated MA mixtures. The results were like those with the 175-μg dose with regard to tumor growth and overall survival times. The relationship between the groups was also similar, and the ultimate cure rates were the same.



TEXT-FIGURE 9.—Three days after establishment of a tumor in the right hind footpad by injection of 10^6 live MA cells, mice were treated with an injection of 175 μg CP (●- - -●), 175 μg CP plus 10^7 irradiated MA (○——○) into the left footpad, 175 μg CP (▲- - -▲) or 175 μg CP plus 10^7 irradiated MA cells (■——■) sc just below the knee of the tumor-bearing leg. Controls were untreated (×——×). *Numbers* are as in text-figure 1.

DISCUSSION

These data show that when CP is mixed with irradiated tumor cells and injected sc, strong, specific, systemic cell-mediated antitumor immunity results. This immunity is demonstrable and effective in a therapy situation; the injection of such mixtures is consistently more effective in either terms of prolongation of survival or number of tumor regressions than injection of CP alone. These findings are in accord with recent studies describing the successful, specific, therapeutic use of local injections of CP-irradiated tumor cell mixtures against a mouse 3-methylcholanthrene-induced fibrosarcoma (Bomford R: Manuscript in preparation). That the immunity arising from the interaction of CP with tumor cells is cell mediated is also evidenced by the recent demonstration that cells from the tumor-draining lymph node exhibited increased cytolytic antitumor activity in vitro after intratumor CP therapy of a mouse mammary carcinoma (*17*).

The CP-mediated anti-MA immunity in this study was reduced in T-cell-deprived mice, which suggests the phenomenon to be at least partially T-cell dependent. Bomford (Manuscript in preparation) also finds that the therapeutic effects of CP-irradiated tumor cell mixtures are considerably reduced in T-cell-deprived mice, and subsequent experiments in this laboratory indicate that the in vitro tumor inhibitory capacity of lymph node cells draining the site of mixture injection in T-cell-deprived mice is less than that of intact mice (Scott MT: Unpublished data). Considerable data have accumulated showing that the effect of CP on various manifestations of T-cell-mediated immunity is one of depression rather than augmentation (*18–22*). In such reports, however, CP was used systemically, and some recent observations concerning the CP-mediated depression of delayed hypersensitivity to sheep red cells show that no depression occurs if CP is injected locally rather than systemically. The impairment of cell-mediated immunity by CP is apparently centered in the spleen (*22*). There are data from nontumor systems which also indicate augmentation of cell-mediated immunity by a local effect of CP. *C. granulosum,* which is similar to CP in its reticuloendothelial stimulatory (*23*) and antitumor properties (*24*), has been shown to have an adjuvant effect on the purely T-cell response to azobenzenearsenate-*N*-acetyl-L-tyrosine when mixed with antigen and injected locally (*25*).

The present study failed to demonstrate any tumor immunity after the systemic administration of CP and irradiated MA cells either iv or ip. The combination of CP iv and irradiated MA sc similarly failed to evoke tumor immunity (*7*). Some other data support the idea that systemic CP does not augment specific tumor immunity. Biweekly therapeutic ip injections of CP and killed mammary adenocarcinoma cells are no more tumor inhibitory than ip injections of CP alone (*26*). Another study showed that CP given ip did not augment the in vitro cytotoxicity of tumor-draining lymph node cells, whereas local CP injection did (*17*). At variance with these results are those reported for a mouse leukemia model in which cytolytic activity of lymph node cells from a tumor-bearing mouse was both enhanced and prolonged by CP given ip (*1*). In keeping with the negative findings, various studies have shown that the in vivo protective effects of systemic CP, against both solid and systemic tumors, are resistant to various immunosuppressive procedures and are therefore probably largely nonspecific and macrophage mediated (*4, 7, 14–16*).

The present data give no insight into the reasons for the apparent failure of development or lack of involvement of specific tumor immunity in systemic CP-MA systems; however, some considerations are apparent. The failure to achieve immunity in the instance of systemic CP and peripherally localized tumor antigen (*7*) may be attributable in part to the demonstrated requirement for lymph node-mediated contact between CP and tumor antigen. Such reasoning does not seem to apply, however, to systemic administration of both tumor antigen and CP, since contact between the two would be expected to occur at various lymphoid levels, especially in the spleen. Another consideration is that immunity arising from splenic and lymph node processing of antigens differs qualitatively (*27*). This is apparently true for the MA system, because the sc injection of irradiated MA was without effect, whereas systemic injection consistently caused tumor enhancement. It may then be that further manipulation of dosage and timing of CP and irradiated MA might meet the exacting requirement for the induction of effective antitumor immunity via the systemic route. Another point of interest is the reported depression of cell-mediated immunity by CP. Thus far, all reported cases of depression have followed the systemic injection of CP (*18–22*), but not after local injection (*22, 25*).

Clearly the local injection of CP-irradiated MA tumor cell mixtures is analogous to an intratumor injection of CP. Some similarities between the present findings and previous results obtained with intratumor injection of CP into an established MA suggest that the CP-mediated generation of tumor immunity may be responsible in part for the local inhibition or destruction of tumor after intratumor injection of CP (*8*). Both phenomena are T-cell dependent. The degree of immunity resulting from CP-irradiated MA mixtures is shown here to be maximal if both CP and irradiated MA interact at the same site. It is reduced but still considerable if contact between the two is mediated by a common draining lymph node, and nonexistent if the two are injected at

distant sites. The same is true for the effects of an sc injection of CP on an established MA: Intratumor injection was the most effective; reduced but marked tumor inhibition occurred if the tumor-draining node was stimulated with CP, but distant injections were without effect (*8*). The present studies show that systemic tumor immunity is reduced with high doses of CP and the same is also true for tumor inhibition after intratumor injection of CP (*8*). The local antitumor effects associated with intratumor injection of CP are, however, clearly not all attributable to specific antitumor immunity. Previous studies indicate a component of them to depend on the development of a specific immune response to CP (*8*), whereas the present CP-mediated generation of tumor immunity is not. Another discrepancy is that, whereas both processes are reduced with high doses of CP, the overall dose responses are different: The optimal inhibiting dose of intratumor CP injected into an established MA was 70 μg, with lower doses considerably less effective (*8*), whereas extremely low doses of CP (3.5 μg) mixed with irradiated cells produced maximal tumor immunity. It seems then that the local antitumor effects of an intratumor injection of CP may be attributed to at least two sources. In addition to the previously described nonspecific "innocent bystander" effect arising from a specific immune response to CP (*8*), there is also the specific immune destruction of tumor resulting from the interaction of CP with tumor antigen.

A cause for concern with any immunostimulation regimen in cancer therapy is the possibility of stimulating undesirable aspects of tumor immunity, i.e., enhancing tumor growth. To date there have been no published reports of enhancement concerning CP and the present findings are similarly encouraging. No enhancing activity was detected in the serum of mice immunized with CP-irradiated MA mixtures sc. Further, the enhancing activity of systemic injection of irradiated MA cells alone was abolished by the simultaneous injection of CP. The spleen cell transfer experiments suggest that this effect is not due to a counteracting development of tumor immunity but is more likely attributable to a nonspecific mechanism, possibly clearance of blocking complexes from the circulation by the activated reticuloendothelial system. These findings agree with those of Proctor et al. (*28*) who also demonstrated abolition of the enhancing effect of irradiated rat hepatoma cells by injection of CP and also suggested that it may be attributable to the nonspecific activity of reticuloendothelial system.

The production of specific, systemic antitumor immunity by local injection of CP–tumor cell vaccine mixtures may be an additional clinical approach to the use of CP. The clinical feasibility of such tumor cell vaccines in combination with BCG has been established (*29*). The therapy experiments described in this paper have been empirical, and further manipulation of the many variables involved, e.g., multiple injections, timing, and alternate means of tumor cell inactivation, may significantly increase the effectiveness of such mixed vaccines.

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Brief Communication: Failure of *Corynebacterium parvum* Presensitization To Modify the Antitumor Effects of Systemic and Local Therapeutic Injections of C. *parvum* in Mice [1, 2]

Martin T. Scott [3, 4, 5]

SUMMARY—The therapeutic effects of iv and intralesional injection of *Corynebacterium parvum* against mastocytoma P815 in mice, presensitized and showing marked delayed hypersensitivity to *C. parvum*, were no different from those in unimmunized controls.—J Natl Cancer Inst 56: 675–677, 1976.

Evidence is accumulating that a component of the antitumor activity of *Corynebacterium parvum* (CP) may be the cell-mediated immune response to CP itself (*1–3*). A similar dependence has been described to underlie the antitumor effects of two other nonspecific bacterial vaccines, BCG (*4, 5*) and *Listeria monocytogenes* (*6*). It was decided, therefore, to investigate whether presensitization of mice to give delayed hypersensitivity (DTH) reactivity against CP would augment the effectiveness of subsequent therapeutic injections of CP against an established tumor. The mouse tumor model, mastocytoma P815 (MA), was used extensively in previous studies (*1, 7–9*) to elucidate the antitumor effects of CP.

MATERIALS AND METHODS

Mice.—Adult (C57BL/6 × DBA/2)F_1 (B6D2F_1) males were used. T cell-deprived mice were prepared by thymectomy, irradiation, and bone marrow reconstitution, as described in (*7*).

CP was provided by Burroughs Wellcome Co., Research Triangle Park, North Carolina.

DTH to CP.—Mice were sensitized by sc injections of 70 μg CP into a hind footpad. DTH was elicited at various times after sensitization by a 70-μg sc injection into the contralateral footpad and recorded as footpad thickness, measured with a dial gauge caliper, 24 hours after the eliciting injection. Results are expressed as the difference in footpad thickness between sensitized mice and normal mice receiving only the eliciting injection of CP. Characteristics of DTH reactivity to CP in B6D2F_1 mice were described in (*1*).

Tumor.—The characteristics of MA and its growth in B6D2F_1 mice were described in (*1, 7, 9*). Solid tumors were established in the hind footpad by an sc injection of 10^6 live MA cells. The footpad size was measured with a dial gauge caliper. Differences in mean tumor size between experimental groups for each time point were compared with the use of a Student's *t*-test.

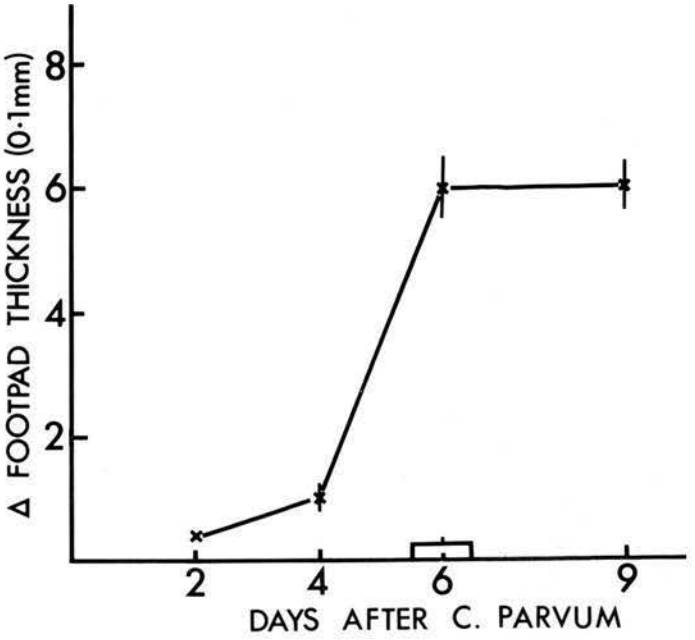
RESULTS

DTH to CP

Mice were sensitized by an sc injection of CP as described, and DTH was elicited at the times shown in text-figure 1. Significant levels of DTH were detected in intact mice 4 days after CP sensitization and were maximal by day 6. No significant DTH reactivity was detectable in T cell-deprived mice 6 days after sensitization.

Effect of CP Presensitization on Intravenous CP Therapy

These experiments were arranged so that the therapeutic injection of CP was 6 days after sensitization, i.e., when mice showed marked DTH reactivity to CP. Both normal mice and those presensitized with 70 μg CP sc into a hind footpad 4 days previously received 10^6 live MA cells sc into the contralateral footpad. Two days later mice from both groups were given either 700 μg CP iv or no treatment (text-fig. 2). Presensitization alone had no effect on tumor growth, and CP given iv caused a marked but transient growth inhibition, which was reflected in a significantly increased mean survival time. Presensitization did not modify the effects of CP given iv, as judged by both tumor growth and survival time. This lack of effect was confirmed in a repeat experiment.



TEXT-FIGURE 1.—DTH to CP: At the times shown, an eliciting dose of CP (70 μg) was injected into the contralateral footpad, and footpad thickness measured at 24 hours (×——×). Results are expressed as the difference in footpad thickness between sensitized mice and unsensitized mice receiving only eliciting CP. Histogram shows DTH reactivity in T cell-deprived mice, 6 days after sensitization.

Effect of CP Presensitization on Intratumor CP Therapy

Mice were given sc injections of 10^6 live MA cells into a hind footpad and, 1 hour later, received an sc sensitizing injection of 70 μg CP into the contralateral footpad

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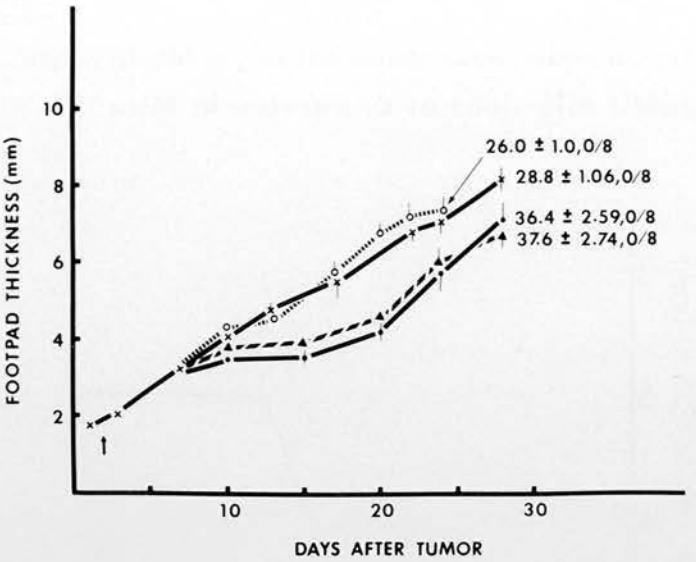
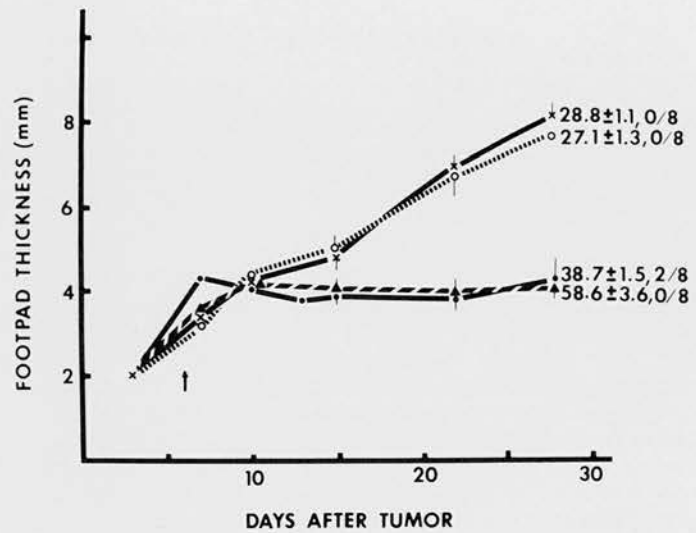
[5] The excellent technical assistance of Sandra L. Warner is gratefully acknowledged.



TEXT-FIGURE 2.—Effect of CP presensitization on intravenous CP therapy. Growth of 10^6 MA cells in the footpads of normal mice (×——×), and mice presensitized 4 days earlier by sc injection of 70 μg CP into the contralateral footpad (○– – –○). CP (700 μg) was injected iv 2 days after tumor establishment in additional groups of both normal (▲– – –▲) and sensitized (●——●) mice. *Numbers in parentheses* represent mean survival time±SE, number of survivors/number in experimental group.



TEXT-FIGURE 3.—Effect of CP presensitization on intratumor CP therapy. Growth of 10^6 MA cells in the footpads of normal mice (×——×) and mice sensitized on the same day by sc injection of 70 μg CP into the contralateral footpad (○– – –○). CP (70 μg) was injected into the tumor, 6 days after tumor establishment in additional groups of both normal (▲– – –▲) and sensitized mice (●——●). *Numbers in parentheses* represent mean survival time±SE, number of survivors/number in experimental group.

or no treatment. Six days later, mice from both groups received 70 μg CP directly into the growing tumor or were untreated (text-fig. 3). Presensitization alone again did not affect tumor growth. In the experiment (text-fig. 3), an intratumor injection of CP in normal mice markedly inhibited tumor growth and prolonged survival time. The immune state of the presensitized mice was apparent from the increased swelling at the tumor site, 1 day after intratumor injection of CP. Presensitization did not modify the inhibition of tumor growth, and the mean survival time of those mice that died was significantly reduced ($P<0.001$). Two complete regressions did, however, occur. In a repeat experiment, the regression was reversed, i.e., 1/8 regression in the normal mice and 0/8 in the presensitized group. In this case, no significant differences were noted in either growth inhibition or mean survival times.

DISCUSSION

It is apparent that the in vivo antitumor effects of CP may be both specifically and nonspecifically mediated [reviewed in (*10*)]. The effects of systemic CP are extremely rapid in onset (*11*) and resistant to various immunosuppressive procedures (*10, 11*); this implies a nonspecific, macrophage-mediated mechanism. Such nonspecific CP-activated macrophage activity is considered the predominant effect of CP given iv against MA (*7*). Recent work (*2, 3*) has shown that, in addition to a direct activation of macrophages by CP, there is an immunologic pathway dependent on T-cell immunity to CP. Activation of macrophages by CP in vitro required the presence of CP-immune spleen cells (*2*), and the onset of macrophage activation by CP in vivo was more rapid in CP-sensitized mice (*3*). The present therapy studies with MA showed, however, no increased effectiveness of CP given iv to mice presensitized and showing DTH reactivity to CP. Two considerations are apparent: *a*) The direct pathway of activation may predominate in vivo, and *b*) any such transient increased reactivity of CP-activated macrophages in CP-sensitized mice, as detected in vitro (*3*), may be insufficient to influence the outcome of a relatively prolonged in vivo situation.

The failure of presensitization to modify the effect of an intratumor injection of CP in the MA system is more impressive, since DTH reactivity to CP occurs directly at the tumor site. Concurrent studies (*12*) in these laboratories with fibrosarcoma Meth-A in a semisyngeneic system similar to the present MA model, clearly show that the local destruction of tumor cells by CP in injected mixtures of CP and live tumor cells completely depends on the local inflammatory response mediated by a DTH response to CP. Regression of such mixtures was enhanced in mice presensitized to CP and, in the case of an intratumor injection into an established solid Meth-A, tumor inhibition occurred only in presensitized mice. The difference between these findings and those with the MA system may be reconciled by the apparent differences in susceptibility of the tumors to the various antitumor components of CP. Unlike Meth-A, local destruction of MA cells only partially depends on the nonspecific activity arising from a local immune reaction to CP (*1*). The remaining activity has been attributed to the CP-mediated generation of specific tumor-immune cells, a process apparently independent of CP immunity for MA (*9*). The failure of CP presensitization to augment the overall effects of an intratumor injection of CP against MA suggests this latter mechanism may be dominant. Additional experiments in the present series (data not included) showed that the specific tumor immunity arising from sc injection of CP-irradiated MA mixtures (*9*) is not modified in mice presensitized to CP.

Clearly, some components of the in vivo antitumor activity of CP arise from the generation and expression of immunity to CP (*1–3, 13*), and the possible modification of the antitumor activity of CP by preexisting immunity to CP may be a relevant consideration in protocols with the use of multiple CP injections. The present

data indicate, however, that, at least for the local destruction of tumor by CP, any such modification may depend on which components of CP-mediated antitumor activity the particular tumor is susceptible to.

A recent study of the local destruction of various tumor cells by BCG in rats similarly concludes that tumors may differ widely in their sensitivity to host reactions aroused by BCG (*13*). Also, two earlier reports describing the mediation of the local antitumor effects of BCG by DTH to BCG in both mice (*5*) and guinea pigs (*4*) failed to detect augmented antitumor activity in animals presensitized to BCG.

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Cancer Immunology and Immunotherapy

Short Communication

C. parvum in Germ-Free Mice*

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Summary. *The effect of previous sensitization to* C. parvum, *by cross-reacting antigens from other bacteria, on the immunostimulatory effects of* C. parvum *treatment were studied in germ-free and conventional mice. It was found that the development of splenomegaly and specific delayed hypersensitivity following* C. parvum *injection were similar in both germ-free mice and conventional mice.*

Introduction

There are data indicating that both the in vitro and in vivo stimulatory and antitumor effects of *C. parvum* depend, at least in part, on the specific immune response to *C. parvum* (Scott, 1974; Christie and Bomford, 1975; Bomford and Christie, 1975; Tuttle and North, 1976). The onset of splenomegaly and macrophage activation has been found to be more rapid in mice presensitized to *C. parvum* (Bomford and Christie, 1975) and, in a particular mouse fibrosarcoma model, the expression of the local antitumor effect of *C. parvum* occurred only in presensitized mice (Tuttle and North, 1975).

Preexisting immunity to *C. parvum* is thus likely to modify the effects of a subsequent injection of *C. parvum*. Recent studies (Woodruff et al., 1974; James et al., 1975; Wolberg et al., 1977) have demonstrated that significant levels of anti-*C. parvum* antibody are present in both normal mouse and human serum, and it has been postulated that these resulted from prior interaction with cross-reacting bacterial antigens. We therefore decided to compare the effect of *C. parvum* in germ-free and conventional mice, in order to determine the role of previous sensitization, from other bacteria, on the immunopotentiating effects of *C. parvum* treatment.

Materials and Methods

Animals. Non-inbred Tru : ICR mice aged 5–8 weeks were used in all experiments. Conventional animals were housed 10 to a cage and maintained on a sterilized diet and acidified water. Sera from these mice all showed positive agglutinating reactivity with *C. parvum*. Germ-free mice were kept in Trexler-type flexibile isolators (Trexler et al., 1957) on sterile hardwood chip bedding and given sterile water and diet RMH 3500 (Agway Inc., Syracuse, N.Y.) and libitum. The gnotobiotic status of the mice was checked regularly during the experiments by following accepted procedures for microbial monitoring (Wagner, 1959). In all cases the mice remained axenic throughout the experiments.

C. parvum. Strain CN 6134 (Wellcome Research Labs. Beckenham, Kent, UK) was diluted to the appropriate concentration in sterile saline.

Delayed Type Hypersensitivity (DTH) to C. parvum. Mice were sensitized by s.c. injection of 700 μg *C. parvum* into the nape of the neck and 10 days later DTH was elicited in a hind footpad by s.c. injection of 10 μg *C. parvum* (0.04 ml volume). Nonsensitized control mice were similarly injected in a hind footpad. 24 h later the thickness of the injected and contralateral footpads were measured using dial gauge calipers (Schnelltaster, H. C. Kroplin, GMBH, Hasson, West Germany). The development and characteristics of DTH to *C. parvum* in mice have been described previously (Scott, 1974; Tuttle and North, 1975).

Splenomegaly. 350 μg *C. parvum* was injected i.v. and spleens and body weights were measured at the times shown. Results are expressed as a spleen index:

$$\frac{\text{Spleen weight (mg)}}{\text{Body weight (g)}}.$$

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Results

Splenomegaly (Fig. 1). The spleens of unimmunized germ-free mice were significantly smaller than conventional mice ($P < 0.01$), but neither the kinetics nor magnitude of the spleen index resulting from i.v. *C. parvum* were significantly different between the germ-free and conventional mice.

DTH to C. parvum (Fig. 2). The nonspecific inflammatory swellings caused by the eliciting dose of *C. parvum* in nonsensitized germ-free and conventional mice were not significantly different, nor were the levels of DTH following *C. parvum* sensitization.

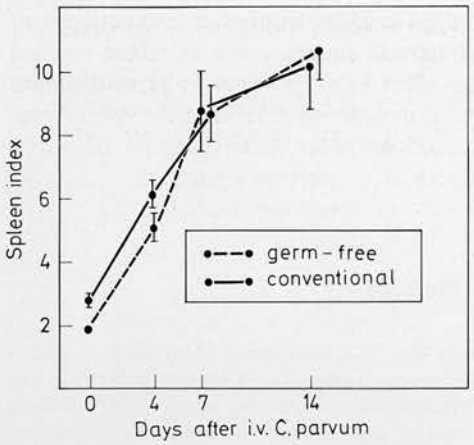

Fig. 1. Effect of an injection of 350 μg *C. parvum* i.v. on the spleen index in germ-free and conventional mice (5 mice per point). ●----● germ-free; ●——● conventional

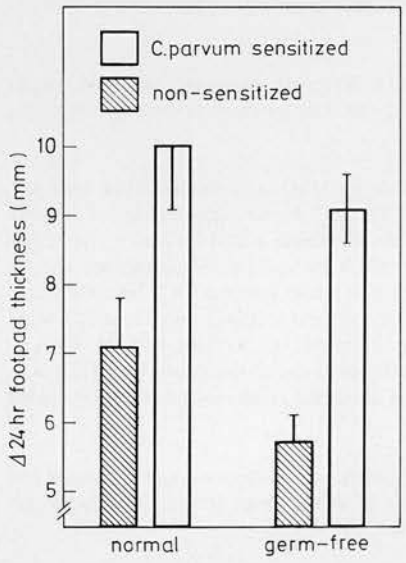

Fig. 2. The DTH response to *C. parvum* in conventional and germ-free mice (5 mice per group). □ *C. parvum* sensitized, ▨ non-sensitized

Discussion

As outlined in the Introduction, it seems that a true primary immune response to *C. parvum* may be unobtainable in conventional mice due to pre-existing immunity resulting from prior interaction with cross-reacting bacteria (Wolberg et al., 1977). We therefore chose to examine the responsiveness of germ-free mice to *C. parvum* reasoning that, since a component(s) of the stimulatory effects of *C. parvum* is considered to result from specific immunity to *C. parvum* (Scott, 1974; Christie and Bomford, 1975; Bomford and Christie, 1975; Tuttle and North, 1975), these effects may be reduced in germ-free animals. *C. parvum* has, however, been found to be fully capable of lymphoreticular stimulation in germ-free mice as evidenced by splenomegaly. The increase in spleen weight following systemic *C. parvum* injection has correlated with antitumor (McBride et al., 1975; Riveros-Moreno et al., 1978) and other potentiating effects (Adlam and Scott, 1973; O'Neill et al., 1973).

The development of cell-mediated immunity to *C. parvum* in germ-free mice was similarly unimpaired, and it is this aspect of *C. parvum* immunity which has been implicated as a basis for its antitumor activity (Christie and Bomford, 1975; Bomford and Christie, 1975; Tuttle and North, 1975). The present data do not of course exclude an immune basis for the stimulatory effects of *C. parvum*, but show that they may be fully established in the absence of previous sensitization.

A further implication of the finding that *C. parvum* is active in germ-free mice concerns the current interest in the application of gnotobiotic techniques in cancer therapy. Germ-free animals will tolerate more cancer chemotherapy than conventional animals (Srivastava et al., 1976), and the effects of chemotherapy can be potentiated by combination with *C. parvum* (Fisher et al., 1976; Purnell et al., 1977). It may therefore be possible to exaggerate such drug-*C. parvum* interactions using gnotobiotic conditions.

Since completion of these studies, a report describing the in vitro tumor inhibitory capacity of *C. parvum*-activated peritoneal cells from germ-free mice as similar to that of *C. parvum*-activated cells from conventional mice has appeared (Fray et al., 1978).

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Comparison of the Potentiation of Specific Tumor Immunity in Mice by *Corynebacterium parvum* or BCG[1, 2]

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ABSTRACT—Two independent studies have compared killed *C. parvum* (CP) vaccine with viable BCG (Pasteur) and BCG (Glaxo) vaccines, respectively, for potentiation of antitumor immunity when injected with irradiated tumor cells into B6D2F$_1$ and CBA-T$_6$ T$_6$ mice. Both studies concurred that, for a given number of irradiated tumor cells, larger amounts (dry weight equivalent) of BCG than CP were required to produce similar degrees of tumor immunity. Evidence also showed that stronger and more protracted immunity was mediated by CP than by BCG.—J Natl Cancer Inst 57: 555-559, 1976.

The modes of antitumor activity of the two bacterial vaccines BCG and CP, currently under clinical investigation as immunotherapeutic anticancer agents, may be similar; each probably potentiates nonspecific macrophage-mediated (*1, 2*) and specific lymphocyte-mediated (*3–5*) antitumor immunity. The distinction between these two types of antitumor resistance has been particularly clearly delineated in the case of CP. Systemic (iv or ip) injection of CP produces a predominantly nonspecific antitumor resistance that is still demonstrable in mice immunosuppressed by T-cell depletion (*6–8*) or irradiation (*9*). Therefore, it probably does not involve a host immune response to tumor antigens and may be mediated by activated macrophages, which appear after CP injection in normal and T-cell depleted mice (*10, 11*). In contrast, the injection of CP directly into a tumor or admixed with tumor cells produces highly specific antitumor resistance only in immunologically intact mice (*4, 12, 13*) and probably involves the promotion of cell-mediated immunity to tumor-specific transplantation antigens (*3, 4*).

Direct comparative data concerning CP and BCG are clearly required. This paper describes two independent investigations in which the specific immunity that results from local interaction of either BCG or CP with irradiated tumor cells are compared. MA, the tumor model used in one of the studies (M.T.S.), in B6D2F$_1$ mice, seemed particularly appropriate for comparative work, inasmuch as results of extensive independent studies in the same laboratory revealed that interaction of irradiated MA cells with CP (*3*) or BCG (*5*) result in specific cell-mediated antitumor immunity. The second tumor model studied was M4, and a similar type of immunity was observed from interaction of irradiated M4 cells and CP (*4*).

MATERIALS AND METHODS

Mice.—Adult male (C57BL/6 × DBA/2)F$_1$ (B6D2F$_1$) and CBA-T$_6$T$_6$ mice were used for the mastocytoma and fibrosarcoma studies, respectively. MA was originally induced by 3-methylcholanthrene in a DBA/2 mouse and has been used extensively in B6D2F$_1$ mice for 3 years. Its maintenance, harvesting, and growth characteristics as a solid tumor in these mice have been described in (*8*). Techniques for the maintenance and harvest of M4, originally induced by 3-methylcholanthrene in a CBA-T$_6$T$_6$ mouse and subsequently maintained in tissue culture, were described in (*4*).

Statistics.—In those tests in which P was less than 0.05 with Student's t-test, results were considered significant.

BCG (Pasteur).—Used throughout the MA studies, Pasteur strain BCG (TMC 1011) from the Trudeau Mycobacterial Collection was grown as dispersed cultures in Middlebrook 7H9 medium containing Tween 80 (Difco, P.O. Box 1058A, Detroit, Mich.). After 7 days of incubation, cultures were frozen slowly in 1-ml vials to −70° C and stored at this temperature (*14*). In some repeat experiments, the same strain of BCG was grown in dispersed cultures in Proskauer-Beck medium (Difco) containing 0.05% Tween 80. After 6 days of culture, the organisms were used without frozen storage. Absolute counts were made with a Petroff-Hauser chamber and phase contrast microscopy. The cultures were plated on Middlebrook 7H10 medium, and viable counts were performed. BCG suspensions were concentrated by our allowing them to settle overnight at 37° C and by removal of the supernatant. Total and viable counts were again recorded. We killed appropriate BCG suspensions by heating them in a waterbath at 60° C for 1 hour; viable counts were performed to ensure complete killing.

BCG (Glaxo).—Batch P658H of "Dried BCG Vaccine (Percutaneous)" (Glaxo Laboratories Ltd., Greenford, England) was used throughout the M4 studies and was resuspended in saline for injection. Mr. T. Varley of Wellcome Research Laboratories did the viable counts by plating the cultures on Dubos' T.B. medium.

CP.—In both studies, killed suspensions of CP (Coparvax, Wellcome Research Laboratories) prepared as previously described (*15*) were used.

Tumor cell irradiation.—MA cell suspensions were exposed to 6,000 rads from a ^{137}Cs source as described in (*3*). M4 cell suspensions were exposed to 10,000 rads from a ^{60}Co source (*4*).

ABBREVIATIONS USED: CP=*Corynebacterium parvum;* MA=mastocytoma P815; M4=fibrosarcoma.

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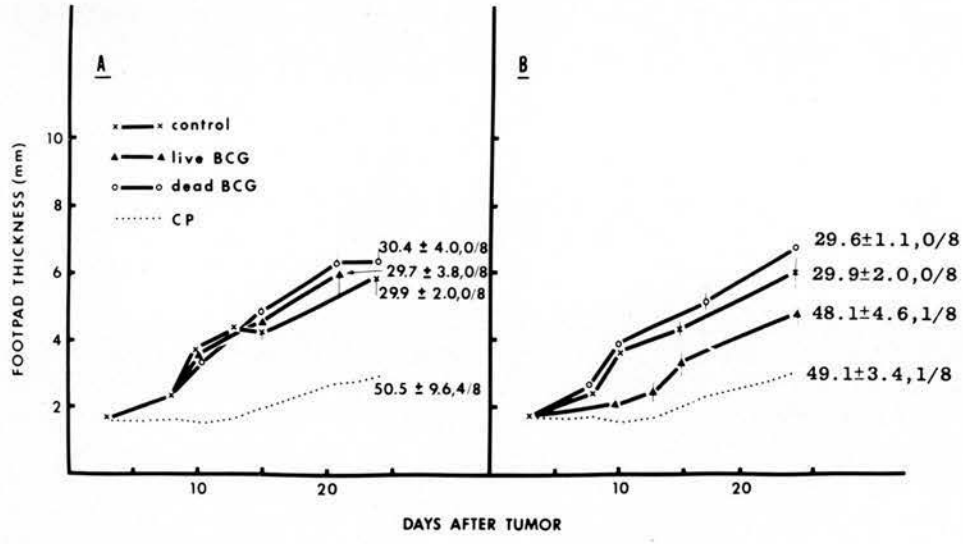
RESULTS

Comparison of CP and BCG (Pasteur) by Use of MA

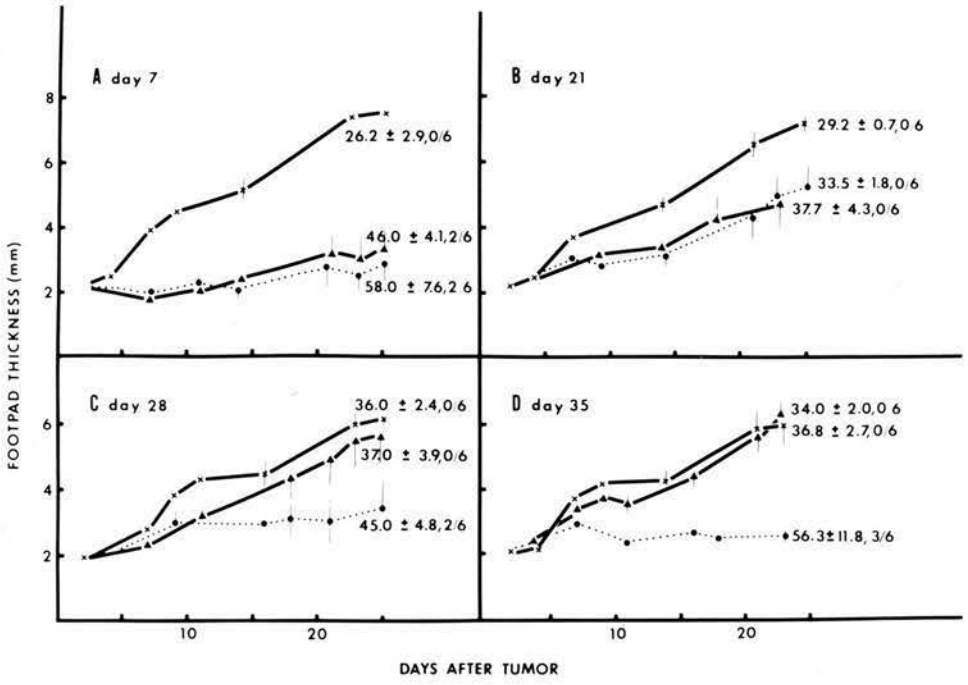
The principle of the experiments with MA was immunization of mice by sc injection into a hind footpad of mixtures of irradiated MA cells and either BCG (Pasteur) or CP. Immunity was assessed by monitoring the growth of a subsequent live MA cell challenge in the contralateral foot. In the first series of experiments, a previously determined (*3*) optimally immunizing dose (3.5 μg) of CP was compared with an equivalent amount of BCG. Mice received 3.5 μg of either live (2×10^6 viable organisms) or heat-killed BCG mixed with 10^7 irradiated MA. CP-treated mice received 3.5 μg CP mixed with 10^7 irradiated MA, and controls were untreated. After 7 days, all animals were challenged with 10^6 live MA cells (text-fig. 1A). CP-treated mice were markedly immune as evidenced by reduced tumor growth, prolonged survival time, and 50% tumor-free animals. With both live and dead BCG, tumor growth was no different from that of untreated controls. A repeat experiment confirmed this lack of effect. Other controls (data not shown) had no reactions from injections of CP, BCG, or untreated MA alone (*3*).

For the second series of experiments, BCG was concentrated as described, and again equivalent dry weights of BCG and CP were compared. Mice were immunized with 10^7 irradiated MA cells mixed with 17.5 μg either live (9×10^6 viable organisms) or heat-killed BCG. CP-treated animals received 17.5 μg of the vaccine mixed with 10^7 irradiated MA cells, and controls were untreated. Live tumor challenge was given 7 days later (text-fig. 1B). Mixtures containing killed BCG caused slight enhancement of tumor growth, with the mean tumor size being significantly larger than in control animals from day 6 onward. However, survival time was not significantly modified. Live BCG was considerably more effective; tumor size was significantly less than both killed BCG and control groups from day 4. Survival time was prolonged, and tumor growth was completely inhibited in 1 animal. CP caused a similar inhibition of tumor growth, there being no significant difference between the overall survival time and number of complete inhibitions of the CP- and live BCG-treated groups. The result suggested, however, that CP-mediated tumor inhibition might be longer lasting than live BCG inasmuch as mean tumor sizes in the BCG group were significantly larger from day 8 onward. The same significant difference in tumor size was observed in a repeat experiment. Throughout these studies, and in keeping with previous recent data accumulated with the MA model (*3*), 17.5 μg was consistently less effective at immunizing than was 3.5 μg CP (12.5% and 50% complete tumor inhibitions, respectively).

To prove that the immunity resulting from CP may be longer lasting than from BCG, we did other tests by challenging mice at various times after immunization. Here, however, BCG immunization was further optimized. Previous studies in our (M.T.S.) laboratories have shown that optimal immunization against live MA challenge resulted from the injection of irradiated MA into a site prepared by BCG 10 days previously, rather than from the injection of mixtures of BCG and irradiated MA (*16*). This method of BCG immunization was compared with the optimal CP-irradiated MA cell mixture as follows: Live BCG (5×10^6 viable organisms) was injected sc into a hind footpad, and 10 days later 10^7 irradiated MA cells were injected into the same site. On the same day, another group received 10^7 irradiated MA cells mixed with 3.5 μg CP into a hind footpad, and mice from both groups were challenged with 10^6 live MA cells in the contralateral footpad at the times shown (text-fig. 2). Control mice received only tumor challenge. Seven days after immunization, both CP- and BCG-treated mice were equally resistant to tumor challenge, there being no significant differences between mean tumor sizes, survival times, and occurrence of complete tumor inhibition. The same was true for mice challenged 14 (data not shown) and 21 days (text-fig. 2B) after immunization. At day 28, however, BCG was considerably less effective than CP and only significantly different from control at day 9 (text-fig. 2C). By day 35, no immunity was detectable in BCG-treated mice, whereas CP-treated mice were still markedly immune (text-fig. 2D).



TEXT-FIGURE 1.—Tumor immunity after injection of irradiated tumor cells mixed with either live or dead BCG or CP. *See* text for details. *Numbers* represent mean survival times±SE of those animals that died; No. of animals tumor free/No. in experimental group.



TEXT-FIGURE 2.—Tumor immunity at various times after injection of CP-irradiated tumor cell mixtures or irradiated tumor cells into BCG prepared sites. Mice were given sc injections of 3.5 μg CP mixed with 10^7 irradiated MA cells into a hind footpad (●·····●) or 10^7 irradiated MA cells into a footpad prepared 10 days previously by sc injection of 5×10^6 viable BCG (▲——▲). Control mice were untreated (×——×). After 7 (A), 21 (B), 28 (C), and 35 (D) days they were challenged with 10^6 live MA cells in the contralateral footpad, and tumor growth was monitored. *Numbers* are as in text-figure 1 legend.

Comparison of CP and BCG (Glaxo) With the M4 Model

The principle of the studies with fibrosarcoma M4 was essentially the same as for MA except that mixtures were given therapeutically, i.e., after tumor establishment, and viable Glaxo BCG was used. Live M4 cells (10^4) were injected sc into a hind footpad, and 2 days later mice were treated in the opposite footpads with 10^6 irradiated M4 cells, with 6, 30, or 150 μg of either CP or BCG (the BCG containing 5×10^6 viable organisms at the 150-μg dose) or with mixtures of irradiated M4 and bacteria (table 1). M4 cells or bacteria given alone did not prevent tumor outgrowth. With CP–M4 mixtures, the low doses of CP (6 and 30 μg) were equally inhibiting to tumor growth and more so than the highest dose (150 μg). This confirms our earlier observation (*4*). The opposite result was obtained with BCG–M4 mixtures, in which the lowest dose (6 μg) was relatively ineffective, but the two higher doses (30 and 150 μg) were almost equal to the low doses of CP.

The effect of a high dose of BCG and a low dose of CP on increasing tumor burdens was also tested. Mice received 10^4, 4×10^4, or 16×10^4 live M4 cells sc in a hind footpad and were treated 2 days later in the contralateral footpad with 10^6 irradiated M4 mixed with either 6 μg CP or 150 μg BCG. Controls were untreated (text-fig. 3). CP and BCG were equally effective against 10^4 live tumor cells. Against 4×10^4 cells, only CP treatment slowed the rate of tumor growth and resulted in lesion-free animals. CP was still inhibitory against 16×10^4 cells, mean tumor sizes being significantly smaller from day 10 onward, but BCG was again without effect.

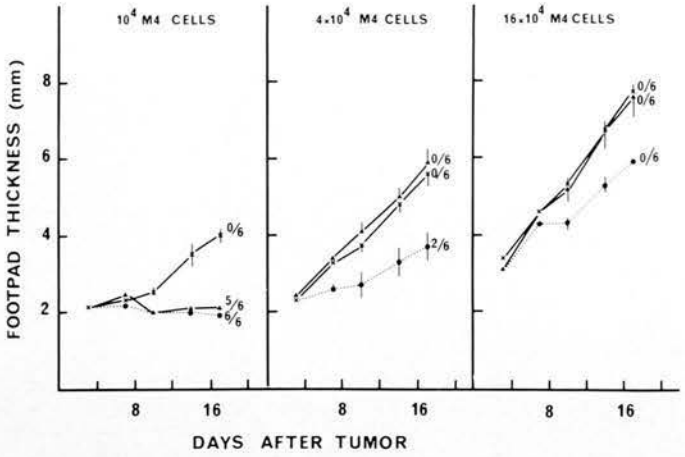
TABLE 1.—*Proportion of mice developing fibrosarcomas after treatment with various amounts of either CP or BCG (Glaxo) with or without irradiated tumor cells*

Treatment	Dosage, μg	Number of mice with tumors/No. treated	
		Without cells	With 10^6 irradiated cells
No bacteria		8/9	11/12
CP	150	4/4	2/4
	30	12/12	0/12
	6	4/4	0/4
BCG	150	11/12	2/12
	30	12/12	2/12
	6	11/12	9/11

DISCUSSION

Using different mouse systems, we have shown in two independent studies that local injection of whole organisms of either viable BCG or killed CP, mixed with irradiated tumor cells, potentiate tumor immunity. Other workers reported that with BCG (*5*) and CP (*3, 4*) the immunity is cell mediated and specific. Here, with MA, heat-killed BCG was ineffective; however, this loss of activity may be a function of the method of killing rather than being indicative of a requirement for living organisms, since recent work established that tumor-specific immunity results from rejection of tumor cells with radiation-killed BCG (*17*) and BCG cell wall–oil droplets (*18*).

It was apparent from the present studies that for a given number of irradiated tumor cells, larger amounts (dry weight equivalent) of viable BCG than killed CP were required to produce similar degrees of antitumor immunity. BCG showed no activity at the low-dose levels at which CP was effective. These and previous studies (*3, 4*) concur that CP inhibited tumor growth maximally at these low doses and, for a constant amount of tumor antigen, was less effective at higher doses. Why CP produces less antitumor immunity is not known. Possible reasons include 1) interference with the function of



TEXT-FIGURE 3.—Therapeutic effect of BCG- or CP-irradiated tumor cell mixtures against increasing tumor burdens. Mice received 10^4, 4×10^4, or 16×10^4 live M4 cells sc in a hind footpad and were treated 2 days later in the contralateral footpad with 10^6 irradiated M4 mixed with either 150 μg BCG (▲——▲) or 6 μg CP (●·····●). Controls were untreated (×——×). *Numbers* represent No. of animals tumor free/No. in experimental group.

the draining lymph node due to intense histiocytic infiltration, 2) the local generation of suppressor macrophages (*19, 20*), or 3) the trapping and retention of responding lymphocytes within the draining node (*21, 22*). With BCG, however, higher amounts were increasingly effective for the dose range studies. When high and low doses of BCG and CP, respectively, were compared in the fibrosarcoma model, CP-mediated immunity was capable of inhibiting larger tumor burdens than was BCG. This may represent a more potent, or more rapid onset of, immunity with CP. That the effects of BCG may be more slowly established is supported by previous findings that tumor immunity arising from BCG-irradiated cell mixtures was less than when the tumor cells were injected into a site prepared by BCG 10 days previously (*16*). This optimal method of BCG-irradiated tumor cell immunization was compared with low doses of CP mixed directly with irradiated tumor cells. Immunity in both groups was similar for 3 weeks after immunization, whereafter BCG-induced immunity was lost but that of CP persisted. Hawrylko and Mackaness (*16*) observed a gradual loss of BCG anti-MA immunity 10 days after immunization.

There have been other reports concerning the use of CP and BCG in the same experimental animal tumor systems but without attempts to compare the activity of equivalent amounts of the vaccines (*23–29*). In some of these studies, the vaccines were used alone and most likely nonspecific immunity mediated by activated macrophages was assessed (*23–26*). With regard to potentiation of specific tumor immunity, mixtures of either CP or BCG with irradiated tumor cells given as multiple local and systemic injections had similar therapeutic effects against lung metastases in rats (*27*). Fisher et al. (*28*) found that 14 days after intralesional injection of 1.4 mg CP into an established mouse mammary carcinoma the cytotoxic activity of regional lymph node cells was potentiated, but not after 0.7 mg BCG (10^7 viable organisms). Specific tumor immunity results from regression of tumors after intralesional injection of CP (*1*) or BCG (*30*) and may be responsible in part for the local inhibition of tumor growth. Intralesional injection of 2 mg *C. granulosum,* which is similar to CP (*15, 31*), caused complete regression of hamster melanomas, whereas BCG ($5–6\times10^6$ viable organisms) only slightly inhibited tumor growth (*29*).

We do not know what determines the relative efficacies of the CP and BCG vaccines used in these studies. The difference may merely represent the relative dilution of active principle(s) by "irrelevant" material, which studies of active fractions, such as have been described for BCG (*18, 32*), should resolve. However, the present direct comparison between killed CP and viable BCG whole vaccines is a practical one because it is in these forms that both are currently undergoing clinical evaluation (*33–36*).

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[CANCER RESEARCH 36, 1335–1338, April 1976]

The Accumulated Effects of Repeated Systemic or Local Injections of Low Doses of *Corynebacterium parvum* in Mice

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SUMMARY

The effects of 14 weekly injections, s.c. or i.v., of "human equivalent" doses (5.25 mg/sq m) of *Corynebacterium parvum* (CP) in mice have been compared. Both s.c. and i.v. CP caused significant splenomegaly and antibody to CP, but stimulation was considerably greater after i.v. CP. Delayed hypersensitivity levels to CP were similar after s.c. and i.v. injection. T-cell competence, as judged by phytohemagglutinin reactivity and delayed hypersensitivity to sheep cells, was unimpaired after s.c. CP and augmented by i.v. CP. Activated peritoneal macrophages capable of nonspecifically inhibiting tumor growth *in vitro* were detected only after i.v. CP, and *in vivo* resistance to tumor cell challenge was greater after CP administered i.v. than s.c.

INTRODUCTION

Most of the data with regard to CP[2] injection in animals have been obtained with high and single doses of CP, as compared with lower but repeated doses used clinically. The currently acceptable dose in humans is around 5 mg/sq m (8, 9, 14), whereas an average dose range for mouse studies has been around 75 to 100 mg/sq m (6) (reviewed in Ref. 19). The present study was undertaken to see whether repeated low (human equivalent) doses of CP resulted in functional alterations in immunity similar to those reported previously for single high doses. The effects of systemic and local CP injections have also been compared, since both routes are used clinically (8, 9, 14).

MATERIALS AND METHODS

Mice. Adult female $CBAT_6T_6$ mice were used throughout except for the carcinogen studies, in which C57BL/6 × DBA/2 F_1 (hereafter called B6D2F_1) mice were substituted. The reactivities of both strains to CP were similar as judged by natural and acquired, humoral, and cell-mediated responses.

Immunization. Mice received injections once a week for 14 weeks, either i.v. or s.c., of 35 μg CP (5.25 mg/sq m) (Coparvax; Wellcome Research Laboratories, Beckenham, Kent, England). The i.v. injections were via the lateral tail vein. The site of s.c. injection was varied because of skin thickening at the site of previous injections, but avoided the right footpad and thigh which were to be the sites of subsequent tumor and carcinogen challenge, respectively. Control mice received no CP.

CP Agglutination. Sera, doubly diluted in isotonic borate-buffered saline, pH 7.2, were dispensed into plastic microtiter trays (Scientific Supplies, Vine Hill, London, England). Where appropriate, mercaptoethanol was then added (0.1 M to a final dilution of 10%), and trays were allowed to stand at room temperature for 30 min. To each well was then added an equal volume of a 1:10 dilution of washed CP. Following brief agitation, trays were incubated for 30 min at 56° and then overnight at 4°. End points were recorded as the reciprocal of the final serum dilution causing agglutination. Duplicate determinations were made on each serum.

DTH to CP. Characteristics of DTH to CP in mice have been described previously (18). Seventy μg CP were injected s.c. into a hind footpad and footpad thickness was measured 24 hr later. Results are expressed as percentage increase in footpad thickness over uninjected feet.

DTH to SRBC. Mice were sensitized by injecting 10^8 SRBC into a hind footpad, and 5 days later DTH was elicited in the ear by s.c. injection of 0.01 ml 50% SRBC. Ear thickness was measured 24 hr later, using the electrical modification of a micrometer screw gauge, as described in Ref. 4.

PHA Response. Spleen cells were prepared and their responsiveness to PHA was assayed as described previously (15). Results are expressed as stimulation indices, (cpm/stimulated culture)/(cpm/control culture).

***In Vitro* Assay for Nonspecific Inhibition of Tumor Growth.** The technique was as described in Ref. 17. Peritoneal cells were mixed *in vitro* at a ratio of 10:1 with CBA-RI leukemia cells (13). Tritiated thymidine was added after 24 hr of culture, and its uptake by tumor cells was assessed 24 hr later.

Tumor Challenge. Mice received s.c. injections into the right hind footpad either s.c. with 2×10^4 syngeneic M4 fibrosarcoma cells (2) or i.p. with 10^2 RI leukemia cells (13). Tumor growth and survival times, respectively, were monitored.

Carcinogen Challenge. Both 3-methylcholanthrene and benzo*(a)*pyrene were suspended in trioctanoin (Eastman Organic Chemicals, Rochester, N. Y.); 1 mg of carcinogen was injected s.c. into the right thigh.

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[2] The abbreviations used are: CP, *Corynebacterium parvum;* DTH, delayed hypersensitivity; SRBC, sheep red blood cells; PHA, phytohemagglutinin; T-cell, thymus-derived lymphocyte.

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most prominent difference between the 2 injection regimens. It is interesting to note that the present finding of unimpaired and augmented T-cell responsiveness to mitogens, after repeated doses of CP s.c. and i.v., respectively, accords with the preliminary clinical data from patients receiving repeated equivalent doses of CP by these routes (14). T-cell competence is clearly an important consideration in CP therapy, since recent animal data show that a component of the antitumor activity of CP is mediated by potentiating specific cell-mediated antitumor immunity (2, 20).

With regard to the choice of route of injection of CP; although it is apparent from recent animal studies that, under some conditions, local injections of CP may be highly effective therapeutically, *e.g.*, intralesional injection (18) or injection near to the tumor site (18, 20), and specific immunotherapy by s.c. injection of CP mixed with irradiated tumor cells (2, 20), given an inaccessible or widely disseminated cancer, the present study suggests that systemic CP may be more effective. The degree of systemic stimulation, especially macrophage activation, achieved by repeated systemic injections of CP was considerably greater than the same doses given locally. Current studies using ^{125}I-labeled CP (M. T. Scott, in preparation) also show a wider distribution of the organisms after systemic injection increasing the probability of CP reaching the vicinity of the tumor and initiating local CP-mediated antitumor effects mentioned above.

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CONDITIONS FAVOURING THE SELECTION OF EITHER SPECIFIC OR NON SPECIFIC *C. PARVUM*-MEDIATED, SYSTEMIC ANTITUMOUR IMMUNITY IN MICE

M. T. Scott

ABSTRACT

Accumulated data suggest that one of two antitumour mechanisms of *C. parvum* may predominate depending on the route of injection of *C. parvum* and its distribution in relation to the tumour. After systemic *C. parvum* the mechanism is considered to be immunologically non specific (i. e. not requiring tumour specific antigens) and mediated by *C. parvum* activated macrophages. After local injection of *C. parvum* the interaction (direct or lymph node mediated) between *C. parvum* and tumour specific antigens has resulted in the generation of immunologically specific T lymphocytes with antitumour activity.

Consideration of the accumulated data concerning *C. parvum* (CP) treatment of various experimental tumours indicates that different effector mechanisms may predominate depending on the route of injection of CP and its distribution in relation to the tumour.

That the antitumour effects of systemically injected (i.v. or i.p.) CP are argely immunologically non specific (i.e. not amplifying a specific antitumour response) is suggested by their resistance to subsequent whole body irradiation (5, 18), and the fact that they are still apparent in athymic mice (32) and mice depleted of T lymphocytes by thymectomy, irradiation and bone marrow reconstitution (23, 31), or antilymphocyte serum (9). Splenectomy, a procedure likely to impair antibody production, did not impair the effects of a subsequent systemic CP injection (6, 14), and systemic CP is also effective in mice genetically selected for low antibody responses (2).

The above characteristics are compatible with macrophage mediated effects and peritoneal macrophages from normal mice injected systemically with CP kill (1, 20), or inhibit the growth and DNA synthesis of a wide range of tumour cells *in vitro* (8, 11, 21), whereas a local injection (s. c.) of CP is considerably less effective (Fig. 1). In addition to peritoneal macrophages, alveolar (21) and spleen (11, 23) macrophages, and, as yet, undefined peripheral blood cells (7) are also effective.

What is the evidence that a non specific destruction of tumour cells by CP activated macrophages occurs *in vivo*? Macrophages are fully activated by CP in both conventionally T cell deprived (4) and nude mice (8), and are also radioresistant (23). These are all characteristics already described for the *in vivo* antitumour effects of systemic CP.

Further implications are that the *in vivo* CP effects are abrogated by macrophage inhibiting agents (15, 20, 25), and that addition of peritoneal macrophages

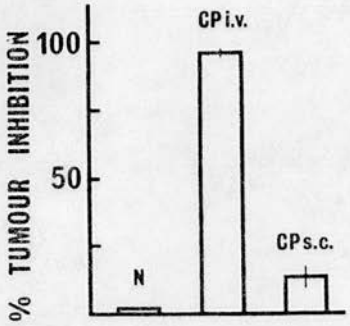

Fig. 1. Percentage inhibition of *in vitro* growth of mouse mastocytoma (P815) cells (see ref 25 for details) by mixing with peritoneal cells from normal mice (N), or mice that had received 700 μg *C. parvum* (CP) either i.v. or s.c. 4 days earlier.

from CP stimulated mice to tumour cells has resulted in the suppression of tumour cell growth when the mixture was injected into syngeneic recipients (Peters et al., this volume). Also, mouse tumours regressing following i.v. CP treatment have been shown to be heavily infiltrated with macrophages (16, 17).

Studies using local injection of CP have revealed a further mode of action which is immunologically specific, i. e. potentiating the host's immune response to tumour specific antigens.

S. c. injection of mixtures of CP and live tumour cells, or direct injection of CP into established tumours has frequently resulted in tumour regressions, and animals whose tumours had regressed were subsequently found to be specifically immune to tumour rechallenge (12, 13, 22). These results suggested that, under conditions of close contact between CP and tumour cells, CP was augmenting a specific antitumour response. Potentiation of specific tumour immunity is also evident from experiments in which subcutaneous injections of CP mixed with irradiated tumour cells have specifically protected mice against both subsequent tumour challenge (24) and established tumours (3, 24), whereas injections of either CP or irradiated tumour cells alone were ineffective. The injection sites of CP and irradiated tumour cells did not need to be coincident for immunity to result, but did require a common lymphoid drainage (24). The immunity arising from local interaction of CP and tumour antigen is cell mediated in that it is reduced in T cell deprived mice (24, 3), and can be transferred by lymph node cells but not serum (24, 27, 29). It may be a relatively short-lived T lymphocyte which probably does not require macrophages or other lymphocytes to effect its antitumour activity (29). That such a cell mediated immune mechanism may operate in the local destruction of tumour cells following direct injection of CP into a tumour site is supported by reports of the T cell dependence of this form of therapy (22, 30). A component of this T cell dependence may, however, also reflect the non specific antitumour activity of macrophages activated locally as a result of an ongoing local T cell dependent immune reaction to CP (28).

The potentiation of specific tumour immunity is not exclusive to locally injected CP, and may be apparent following systemic CP treatment of tumours (33). This seems logical, since systemic CP is widely distributed throughout the lymphoreticular elements of the body following systemic injection (Scott, this volume), and some direct, or lymphoid mediated, CP-tumour antigen interaction is likely to occur.

The ultimate goal of immunotherapy should be the establishment of systemic antitumour immunity since it is metastases persisting, or appearing after successful treatment of the primary tumour that are often the ultimate cause of death. The fact that with CP therapy either specific or non specific systemic immunity may be preferentially selected under different circumstances is a relevant consideration in deciding how it should be used. The intensity and durability of the specific cell mediated antitumour immunity that has resulted from local interaction of CP and tumour antigen in mice (26) suggests this may be a desirable situation to achieve clinically. Optimal immunity may, however, be restricted to situations where either direct intralesional injection, or stimulation of tumour draining nodes is practical, or specific active immune therapy using CP mixed with attenuated tumour cells when these are obtainable. A reasonably strong tumour associated antigen and high degree of patient immunocompetence would both be predicted to facilitate specific immunization.

The immunologically non specific, macrophage mediated, systemic antitumour activity that seems to predominate after systemic injections of CP does not require close contact between CP and tumour, and is likely to be independent of tumour antigenicity and patient immunocompetence. Theoretically, therefore, tumours at inaccessible sites, those of low antigenicity, and patients with poor immunocompetence are situations where non specific therapy may be appropriate.

A further consideration in the selection of specific or non specific CP antitumour effects is the susceptibility of the tumour in question. There are recent animal data showing systemic CP treatment to be relatively ineffective in T cell deprived mice (19), and ineffective in nude mice (10). Macrophages seem to be fully activated in such mice (4, 8) and it thus seems that particular tumours may be insensitive to this form of attack.

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General discussion

McBride: You have nicely shown that intratumoural injection of *C. parvum* gives rise to regression and leads on to a state of specific immunity. Do you know what the level of immunity is in your normal mice not treated with *C. parvum*? What I am really asking is whether you are increasing T-cell immunity or whether you are increasing the expression of T-cell immunity within the tumour.

Scott: We have not examined specific immunity in non-*C. parvum* treated, tumour-bearing mice.

McBride: You haven't tried any transfer experiments?

Scott: No.

Chare: I think that for the sake of completeness we may also mention the work of Fischer in Pittsburgh who has shown that systemic administration of *C. parvum* to tumour-bearing animals does lead to the production of macrophages specifically cytotoxic to the immunising tumours.

Scott: Other workers too have reported potentiation of specific immunity following systemic *C. parvum*.

Gauthier-Rahman: I want to comment on the experiment suggested by Dr. Scott which has a parallel in what we have done with simple protein antigens. The results are very similar. In both cases there is a marked effect due to the route by which you administer *C. parvum*. We found the strongest immunisation was obtained when *C. parvum* is mixed locally with antigen. We used the guinea pig and also did transfer experiments in cell mediated immunity induced by local injection of tumour with *C. parvum* against a tumour specific antigen.

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The Distribution and Persistence *in Vivo* of *Corynebacterium parvum* in Relation to Its Antitumor Activity

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SUMMARY

Killed *Corynebacterium parvum* was labeled with fluorescein isothiocyanate or ^{125}I, and both preparations were shown to retain lymphoreticular stimulatory and antitumor activity. Large amounts of *C. parvum* injected i.v. were found in the liver, spleen, and lungs with less in bone marrow and lymph nodes. Apart from a rapid loss from the lungs within 24 hr, the persistence of killed *C. parvum* was striking, and some intact bacteria were still detectable in the liver and spleen at 15 days. (By contrast, the breakdown of an inactive *C. parvum* strain in the liver was considerably faster.) The blood clearance of ^{125}I-labeled *C. parvum* injected i.v. into tumor-bearing mice was more rapid than in normal mice, and the absolute, but not the unit, amounts of *C. parvum* taken up by the spleen and tumor-draining node were increased. ^{125}I-labeled *C. parvum* was found within the body of established solid tumors, but there was no correlation between the amount of *C. parvum* taken up by various mouse solid tumors after i.v. injection and their susceptibility to i.v. *C. parvum* therapy. The distribution and persistence of *C. parvum* injected into a tumor lesion was similar to that after s.c. injection. The bulk of the inoculum was retained at the injection site and draining lymph node. Contralateral nodes were unlabeled, and uptake in the liver and spleen was considerably less than after i.v. injection. Although no *C. parvum* was found in peritoneal cells after i.v. injection, the macrophages in this population became activated and were capable of nonspecifically inhibiting tumor cell growth *in vitro*.

INTRODUCTION

The bacterial vaccine *Corynebacterium parvum* is an effective antitumor agent in animals (reviewed in Ref. 23) and is currently undergoing clinical investigation (12, 20). The present studies using labeled *C. parvum* preparations were undertaken to delineate the distribution of injected *C. parvum*, which might provide a guideline in selecting the route and site of injection for given tumor situations and further information as to underlying antitumor mechanisms. This paper describes the preparation of both ^{125}I- and fluorescein-labeled *C. parvum* and their distribution after either local or systemic injection in normal and tumor-bearing mice.

MATERIALS AND METHODS

Mice. Adult female CBAT6T6 and DBA/2 mice were maintained at Wellcome Research Laboratories, Beckenham, England. Adult female C3Hf/Bu mice were from the specific pathogen-free breeding colony at the Section of Radiotherapy, M. D. Anderson Hospital, Houston, Tex.

C. parvum. A formol-killed suspension, Coparvax (Wellcome Reagents Ltd., Beckenham, Kent, England), Batch Px401 (7 mg/ml) was used. In some experiments an inactive strain of *C. parvum*, CN5888, was used. It was grown and a formol-killed suspension was prepared as described in Ref. 1.

^{125}I-labeled *C. parvum*. *C. parvum* (5 ml) was washed 2 to 3 times in 0.9% NaCl solution and resuspended in 0.4 ml phosphate-buffered saline (8.01 g NaCl, 1.42 g Na_2HPO_4, 0.41 g KH_2PO_4, and distilled water to 1.0 liter). ^{125}I (2 mCi) (Radiochemical Centre, Amersham, England) was added, followed immediately by 0.4 ml chloramine T (5 mg/ml). After 5 min, 0.4 ml sodium bisulfite (25 mg/ml) was added, followed immediately by 0.4 ml sodium iodide (10 mg/ml). The suspension was washed once by centrifugation in 0.9% NaCl solution and resuspended to its original volume. It was dialyzed for 5 days against daily changes of 2 liters of 0.9% NaCl solution at 4°, by which time the radioactivity in the dialysate had stabilized. In different batches only 0.01 to 0.02% of iodine remained unbound, and no appreciable amount was released after incubating the labeled *C. parvum* for 24 hr at 37°. One-tenth ml of 10% thiomersalate preservative per 10 ml suspension was added. Suspensions were stored at 4°. Concentration was determined by optical comparison with dilutions of unlabeled *C. parvum* vaccine of known concentration. Unless otherwise specified, injections were of 300 μg.

Gamma Counting. Organs or blood samples (50 μl) taken from ^{125}I-labeled *C. parvum*-injected mice were placed directly into plastic tubes and counted using a Packard Tri Carb Automatic gamma counter. Results are expressed as percentage of total isotope injected. Means and standard errors were calculated for all experimental groups, and differences where $p < 0.05$ (Student's t test) were considered significant.

Phagocytic Index (K). Mice received i.v. injections of ^{125}I-labeled *C. parvum* and were bled repeatedly (50-μl samples) at recorded times over a 10-min period. The radioactivity of the samples was determined, and the phagocytic index was calculated as described previously (3).

FITC-CP.[3] *C. parvum* was washed 3 times by centrifuga-

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[3] The abbreviations used are: FITC-CP, fluorescein-labeled *Corynebacterium parvum*; FITC, fluorescein isothiocyanate.

tion in 0.85% NaCl solution and resuspended to its original volume. Buffer was prepared by mixing Na_2CO_3 (53 mg/100 ml) with $NaHCO_3$ (4.2 mg/100 ml) to pH 9.0. One volume of buffer was then added to 9 volumes of washed *C. parvum*. The suspension was set up on a stirrer, and FITC, 1 mg/ml suspension, was added very slowly while stirring at room temperature, and stirring continued for 1.5 hr. The suspension was washed 4 to 5 times in 0.85% NaCl solution to remove unbound FITC and resuspended to its original volume in 0.85% NaCl solution. FITC-CP was stored in the dark at 4° with no noticeable loss of fluorescein into the supernatant during a 1-year period. Unless otherwise specified injections were of 300 μg.

Fluorescent Microscopy. Organs removed from FITC-CP-injected mice were immediately frozen using Polar Spray BPC (dichlorodifluoromethane; Aerosol Marketing and Chemical Co. Ltd., London, England). Cryostat sections were cut at −20°, fixed in methanol for 10 min, and mounted in Polarfluor B (Polaron Experiment Ltd., Watford, England). Blood smears were air dried before fixing and mounting. Peritoneal and bone marrow cells were washed and resuspended at 2×10^6/ml in Eagle's minimum essential medium. Aliquots (0.1 ml) were placed on coverslips, and the cells were allowed to settle for 1.5 hr at 37° in a 5% CO_2 atmosphere. The monolayers were then fixed and mounted as above. Slides could be stored in the dark at 4° for up to 2 weeks without marked loss of fluorescence. They were examined using a Leitz Ortholux II phase contrast/fluorescence microscope.

Tumors. CBA, methylcholanthrene-induced fibrosarcoma cells (M4) were maintained *in vitro* and prepared as described in Ref. 5. Similar details have been given for CBA T3 fibrosarcoma (7) and CBA RI leukemia cells (18). DBA/2 mastocytoma P185 (MA) was obtained in frozen form from Dr. A. C. Allison, Clinical Research Centre, Harrow, Middlesex, England. Cells were maintained by serial ascitic passage and obtained for injection by peritoneal lavage. C3Hf/Bu fibrosarcoma cells (Fsa) were maintained and prepared for injection as described in Ref. 16. Solid tumors were established by s.c. injection of cells into either the hind footpad or flank. Tumors growing in the flank were measured as 2 diameters at right angles, the size being expressed as the multiplicand.

***In Vitro* Assay for Nonspecific Inhibition of Tumor Growth.** The technique has been described before (24). Peritoneal cells were mixed *in vitro* at a ratio of 10:1 with RI leukemia cells. [^{3}H]Thymidine was added after 24 hr of culture, and its uptake by tumor cells was assessed 24 hr later.

RESULTS

Immunostimulatory and Antitumor Activity of Labeled *C. parvum* Preparations. Experiments were performed to confirm that both the FITC-CP and ^{125}I-labeled *C. parvum* preparations retained their lymphoreticular stimulatory and antitumor activity. CBA mice received i.v. injections of FITC-CP, ^{125}I-labeled *C. parvum*, or unlabeled *C. parvum* (Table 1), and, after 14 days, there were no significant differences among the splenomegalies induced by any of the *C. parvum* preparations (Table 1). They were also equally effective when injected into a tumor lesion, all treated tumors completely regressing with no regrowth during a 40-day period. By this time untreated mice were dying with large tumors (Table 1).

Table 1

Comparison of the splenomegaly and tumor-inhibitory effects of unlabeled, FITC-labeled, and ^{125}I-labeled C. parvum, after i.v. and intratumor injection, respectively

Injection i.v. at Day 0	Spleen Wt at Day + 14 (g ± S.E.; n = 4)
	0.08 ± 0.01
C. parvum (300)[a]	0.40 ± 0.06[b]
FITC-CP (300)	0.48 ± 0.06[b]
^{125}I-labeled *C. parvum* (300)	0.39 ± 0.02[b]
Intratumor injection at Day +3[c]	**Proportion of mice tumor free at Day +40**
	0/6
C. parvum (70)	6/6
FITC-CP (70)	6/6
^{125}I-labeled *C. Parvum* (70)	6/6

[a] Numbers in parentheses, dose in μg.

[b] Not significantly different from similarly designated results ($p > 0.05$).

[c] M4 fibrosarcoma cells (10^6) were injected s.c. into a hind footpad, and 3 days later *C. parvum* was injected s.c. into the tumor site.

The Use of FITC-CP. FITC-CP preparations were highly fluorescent (Fig. 1*a*). After injection, fluorescence was clearly visible in tissue sections under ×20 magnification, and, using ×1000 magnification, individual bacteria could be resolved (Fig. 1*b*). Results obtained with FITC-CP provide unequivocal evidence of the presence of *C. parvum* organisms, but it is not possible to quantitate the amount of *C. parvum* in a tissue sample. For quantitative studies of the distribution and persistence of *C. parvum* in organs and other solid tissues, we have used ^{125}I-labeled *C. parvum*, but all experiments were repeated using FITC-CP to confirm the presence or absence and the relative distribution of *C. parvum*. FITC-CP was particularly useful with cell populations (*e.g.*, bone marrow, peritoneal) when FITC-CP-containing cells were readily identified and quantified.

Blood Clearance of ^{125}I-labeled *C. parvum* and FITC-CP in Normal and Tumor-bearing Mice. M4 cells (10^6) were injected s.c. into the hind footpad of CBA mice; 3 weeks later, when the footpad thickness was 7 to 8 mm, the mice received i.v. injections of ^{125}I-labeled *C. parvum*, and the phagocytic index (K) was determined. The clearance of ^{125}I-labeled *C. parvum* from the blood of tumor-bearing mice was significantly faster than normal; $K = 0.25 \pm 0.009$ and 0.14 ± 0.007, respectively. Twenty-four hr after i.v. ^{125}I-labeled *C. parvum* treatment in both normal and tumor-bearing mice, there was no detectable difference between the radioactivity of whole-blood samples and those in which cell and bacteria had been removed by high-speed centrifugation (10,000 × *g* for 15 min). This suggested that *C. parvum* had been completely cleared from the blood within 24 hr. It was confirmed by the complete absence of FITC-CP in blood smears taken 24 hr after i.v. FITC-CP treatment,

whereas after 1 hr some smears still contained FITC-CP-containing cells.

Distribution and Persistence of Labeled *C. parvum* Injected i.v. into Normal and Tumor-bearing Mice. ^{125}I-labeled *C. parvum* was injected i.v. into normal and tumor-bearing CBA mice as above (Chart 1). At the time of injection, spleen weights in tumor-bearing mice (0.19 ± 0.2 g) were significantly larger than normal (0.10 ± 0.004 g), as were tumor-draining lymph nodes: 15.7 ± 0.5 and 1.4 ± 0.3 mg, respectively. There were no significant differences in lung and liver weights.

In normal mice large amounts of radioactivity (80.8% injected) were present in the liver after 1 hr. The levels then declined gradually with 10% still being detected after 15 days. The presence of the tumor did not modify the uptake or persistence of radiaoctivity in the liver. Radioactivity (2.8%) was present in normal spleen after 1 hr and persisted similarly to that in the liver. Significantly more radioactivity (4.8%) was found in tumor spleens after 1 hr; however, the uptake per g spleen was not significantly different: 30.4 ± 4.3% and 30.0 ± 3.0%/g for normal and tumor spleens, respectively. Normal lungs contained 3.8% radiaoctivity at 1 hr, and this declined rapidly over the next 24 hr (0.2%), but more gradually thereafter. Lungs from tumor-bearing mice were not significantly different. Significant levels of radiaoctivity were detectable in normal popliteal nodes only after 1 hr but persisted in tumor-draining nodes for 3 days. Despite the larger amounts of radioactivity in tumor-draining nodes at 1 hr (0.56 ± 0.003% compared with 0.009 ± 0.004%), compensating for their increased mass, the unit uptake (0.004 ± 0.001%/g), was significantly less than in normal and tumor-bearing mice. As noted in the previous section, radiaoctivity in the blood after 24 hr is in soluble form and most probably reflects the breakdown of ^{125}I-labeled *C. parvum* in the various organs.

Parallel studies using i.v. FITC-CP confirmed the presence of intact FITC-CP in the various organs and their relative distribution (semi-quantitative estimate of numbers of FITC-CP per unit section). A correlation between the amounts of FITC-CP and radiaoctivity was particularly apparent from the marked loss of FITC-CP from the lungs between 1 and 24 hr. FITC-CP detected in the liver and spleen after 15 days appeared intact and was similar to that seen after only 1 hr (Fig. 1*b*). Intact FITC-CP have been detected in liver sections up to 28 days after injection.

Distribution and Persistence of Labeled *C. parvum* Injected s.c. into Normal Mice or Intralesionally into Tumor-bearing mice. Injection s.c. of *C. parvum* has been compared with injection of *C. parvum* directly into tumor tissue. The latter has been a particularly effective form of *C. parvum* therapy in many animal tumor models (13, 19, 23, 26) including the present M4 (see Table 1). Normal CBA mice received ^{125}I-labeled *C. parvum* s.c. into a hind footpad. Another group of mice bearing a 3- to 4-mm M4 tumor in the hind footpad received ^{125}I-labeled *C. parvum* s.c. directly into the tumor lesion (Chart 2). The bulk of the radioactivity (75%) was found at the injection site after both s.c. and intralesional injection. It persisted as shown with 0.4 and 0.6%, respectively, remaining at 15 days. The amounts of radioactivity and their persistence in liver and spleen were similar after both injections and were considerably less than after systemic injection (Chart 1). The radioactivity levels in the draining popliteal nodes after both injections were similar to each other and to those found in the spleen. No significant activity was detected in contralateral nodes. A consistent difference in the distribution of radiaoctivity after either s.c. or intralesional injection was found after 1 hr; with s.c. injection, peak levels in the liver, spleen, and draining node were delayed until 24 hr. In both cases these levels paralleled those in the blood. The presence or absence of *C. parvum* in the various organs at different times after both s.c. and intralesional injection was confirmed using FITC-CP.

Localization of i.v.-injected *C. parvum* within a Solid Tumor. Solid tumors were estalished in the flanks of CBA mice by s.c. injection of 10^6 M4 cells; 7 days later, when the tumors were 25 to 30 sq mm and still responsive to i.v. *C. parvum* (see Chart 3), FITC-CP was injected i.v. Twenty-four hr later the tumors were dissected out and carefully cleared of nontumorous tissue. Cryostat sections were prepared

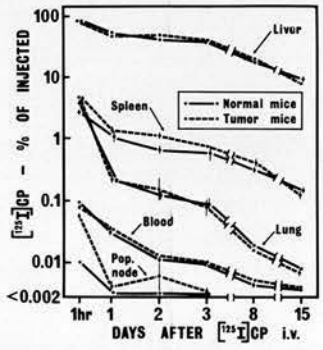

Chart 1. Amounts of ^{125}I-labeled *C. parvum* (*[^{125}I]CP*) in various organs and 50-μl blood samples at various times after i.v. injection into normal mice or mice bearing a solid M4 tumor in a hind footpad (10^6 M4 cells s.c. 3 weeks earlier). *Pop. node*, popliteal node.

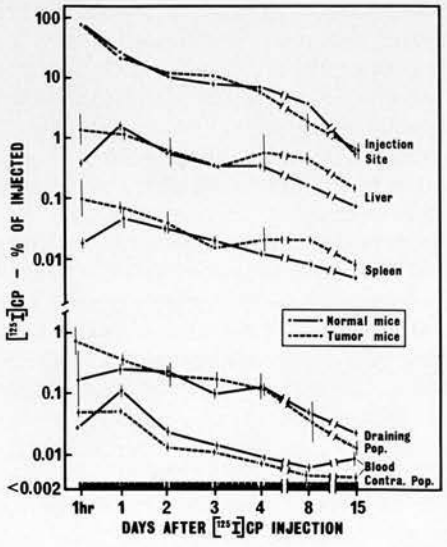

Chart 2. Amounts of ^{125}I-labeled *C. parvum* (*[^{125}I]CP* in various organs and 50-μl blood samples at various times after s.c. injection into a hind footpad of normal mice or intralesional injection into mice with a solid M4 tumor growing in a hind footpad (10^6 M4 cells s.c. 1 week earlier). *Pop.*, popliteal *Contra.*, contralateral.

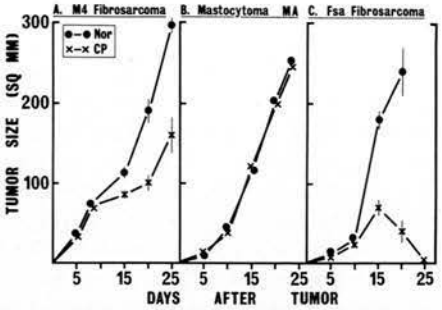

Chart 3. The relative sensitivities of various mouse solid tumors to i.v. *C. parvum* therapy. M4 or MA cells (10^6) were injected s.c. into the flank of CBA or DBA/2 mice, respectively. Fsa cells (5×10^6 were similarly injected into C3Hf/Bu mice. Seven days later, 700 μg *C. parvum* (*CP*) were injected i.v. Control mice (*Nor*) were untreated. Tumor size was measured as 2 diameters at right angles and expressed as the multiplicand.

and examined for the presence of FITC-CP. Intact FITC-CP were visble in all sections. The experiments were repeated using i.v. ^{125}I-labeled *C. parvum*, and 0.007 ± 0.001% of injected radioactivity was found per mg of tumor tissue.

Relationship between Localization of *C. parvum* within a Solid Tumor after i.v. Injection and Ultimate Response to i.v. *C. parvum* Therapy. To determine whether the susceptibility of various solid tumors to i.v. *C. parvum* therapy was related to the amount of *C. parvum* that localized within the tumor, 3 different mouse tumor models were used. The relative sensitivities of these tumors to i.v. *C. parvum* are shown in Chart 3. M4 or MA cells (10^6) were injected s.c. into the flanks of CBA or DBA/2 mice, respectively. Fsa cells (5×10^6) were injected at the same site in C3Hf/Bu mice. *C. parvum* (700 μg) was injected i.v. 7 days later. Growth of MA was completely unaffected by i.v. *C. parvum*, but with M4 there was transient growth inhibition with no complete regressions. In accordance with previous findings (15), after a short period of growth Fsa tumors underwent complete regression. In parallel experiments ^{125}I-labeled *C. parvum* was injected i.v. 7 days after tumor establishment. The tumors were removed 1 and 2 days later, cleared of surrounding tissue, weighed, and counted for radioactivity (Chart 4). The amounts of ^{125}I-labeled *C. parvum* taken up per mg of MA and Fsa were not significantly different and were significantly less than that taken up by M4. Thus there was no correlation between the amount of *C. parvum* that localized within the solid tumors after i.v. injection and the ultimate outcome of i.v. *C. parvum* therapy.

Uptake of FITC-CP by Peritoneal and Bone Marrow Cells. Normal CBA mice received FITC-CP i.v., i.p., or s.c., and the numbers of FITC-CP-containing cells in bone marrow and peritoneal cell preparations were counted (Chart 5). Of bone marrow cells, 10% contained FITC-CP 1 hr after i.v. injection, dropping to 2% after 10 days. A few (approximately 0.1%) FITC-CP-containing bone marrow cells were found in occasional preparations after s.c. and i.p. injection. No FITC-CP-containing peritoneal cells were found at any time after i.v. or s.c. injection. That the peritoneal cells were, however, capable of taking up FITC-CP was apparent from the large number (50%) of FITC-CP-containing cells found after i.p. injection. Despite the presence or absence of FITC-CP in peritoneal cells after i.p. or i.v. injection, respectively, 2 days after injection both cell populations

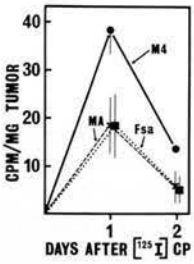

Chart 4. Relative amounts of ^{125}I-labeled *C. parvum* ([^{125}I]*CP* taken up by various mouse solid tumors after i.v. injection. Mice bearing 7-day solid tumors in the flank (see Chart 4) received i.v. injections of ^{125}I-labeled *C. parvum*, and the tumors were removed 1 and 2 days later for radioactive assay.

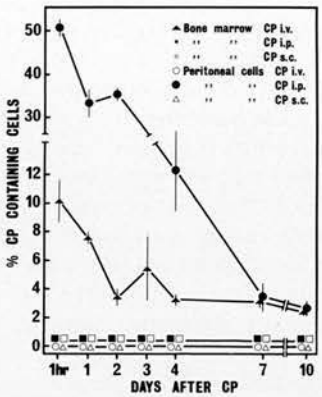

Chart 5. The percentage of FITC-CP (*CP*)-containing cells in the peritoneum and bone marrow at various times after i.v., i.p., or s.c. injection.

Table 2

In vitro inhibition of RI leukemia cell growth by peritoneal cells from mice pretreated either i.v. or i.p. with FITC-CP

Cells	[^{3}H]Thymidine incorporation (cpm/culture[a] ± S.E.)	cpm/culture (% RI alone)
RI alone	32,996 ± 300	100.0
Normal peritoneal cells + RI (10:1)	39,902 ± 3,759	120.9
i.v. FITC-CP peritoneal cells[b] + RI (10:1)	734 ± 96[c]	2.2
i.p. FITC-CP peritoneal cells[d] + RI (10:1)	860 ± 76[c]	2.6

[a] Cultures of normal or FITC-CP-stimulated peritoneal cells alone incorporated insignificant amounts of [^{3}H]thymidine.

[b] Peritoneal cells from mice that had received 300 μg FITC-CP i.v. 4 days earlier.

[c] Not significantly different from similarly designated results ($p > 0.05$).

[d] Peritoneal cells from mice that had received 300 μg FITC-CP i.p. 4 days earlier.

showed similar degrees of macrophage activation as evidenced by degree of spreading and vacuolation (19). Four days after injection the cell populations were compared for their ability to inhibit nonspecifically the growth of syngeneic leukemia cells *in vitro*, and both were found to be highly active (Table 2), with 97.8 and 97.4% inhibition after i.v. or i.p. FITC-CP, respectively.

Failure of an Inactive Strain of *C. parvum* to Persist *in Vivo*. Throughout these studies we were impressed with the relative resistance of *C. parvum* to degradation *in vivo*, some intact FITC-CP still being detectable more than a

month after injection. To determine whether the property may be related to the antitumor activity of *C. parvum*, the *in vivo* persistence of either ^{125}I- or FITC-labeled inactive *C. parvum* (CN5888) was studied. This organism has no lymphoreticular stimulatory or antitumor effects (1) and did not acquire any after labeling. The results of i.v. injection of equivalent amounts of active and inactive ^{125}I-labeled *C. parvum* into CBA mice are shown in Table 3. Considerably more radioactivity was lost from the liver and spleen during the 1st 24 hr after injection of inactive ^{125}I-labeled *C. parvum* than with active ^{125}I-labeled *C. parvum*. Studies using inactive FITC-CP again suggested a rapid breakdown of this organism *in vivo:* 24 hr after i.v. injection no intact bacteria could be resolved in spleen and liver sections, the only fluorescent material resembling debris (Fig. 1, *c* and *d*).

DISCUSSION

^{125}I- or FITC-labeled *C. parvum*, injected i.v. into normal mice, was found in high concentrations in the liver, spleen, and lung, and in lesser amounts in the lymph nodes and bone marrow cells. After s.c. administration, however, most of the bacteria were restricted to the site of injection, with high concentrations in the draining lymph nodes and none in the contralateral nodes. Amounts in the spleen, liver, and bone marrow were far less than after i.v. injection. Although *C. parvum* was rapidly lost from the lungs after i.v. injection, it was remarkably persistent elsewhere; this will be discussed later. The presence of *C. parvum* in the various tissues corresponds with reported biological modifications in them after injection of *C. parvum, e.g.,* hepatomegaly, splenomegaly and lung weight increase (1), increased lymph node weights (8), augmented proliferative response of lymph nodes to mitogens (14) and antigens (25), and increased colony-forming capacity of bone marrow cells (30).

The restricted distribution of *C. parvum* after s.c. injection results in intense stimulation of the draining lymph nodes with only minimal splenomegaly and hepatomegaly compared with the i.v. route. This suggests that the degree of regional stimulation caused by *C. parvum* may be directly proportional to the amount of *C. parvum* reaching the tissue. The relative distributions of *C. parvum* after these routes of injection also parallel the *in vivo* antitumor activities induced. Whereas systemic *C. parvum* is capable of inhibiting the growth of tumors at various sites in the body (reviewed in Ref. 23), s.c. *C. parvum* is usually only effective when injected either directly into the tumor or so that it stimulates the tumor-draining nodes (13, 26). The same relationship holds for the generation of specific cell-mediated tumor immunity after local injections of *C. parvum* and irradiated tumor cells (5, 27). The immunity resulting from injecting irradiated tumor cells and *C. parvum* at separate sites having the same draining lymph node is as strong as when they are injected as a mixture, but is reduced by wide spatial separation of the injections (27).

The presence of a solid fibrosarcoma growing in the footpad increased the rate of clearance of i.v. *C. parvum* from the blood, and this elevated phagocytic activity of tumor-bearing mice has been noted previously (17). The amounts of *C. parvum* taken up by the spleen and tumor-draining node were also increased, but liver and lung uptake were not modified. That the increased splenic uptake was proportional to the tumor-induced splenomegaly suggests an increased nonspecific trapping of *C. parvum* rather than any selective uptake attributable to the tumor. This also applies to the increased uptake by the tumor-draining node where the unit uptake was actually decreased. However, the increased total amount of *C. parvum* in the tumor-draining node may contribute to the efficacy of i.v. *C. parvum* therapy, since stimulation of a tumor-draining node by *C. parvum* is known to result in specific cell-mediated antitumor immunity, whereas distant injections are ineffective (27).

The distribution of *C. parvum* after intralesional injection resembled that after s.c. injection into normal animals. However, the systemic spread of *C. parvum* was more rapid after intralesional than after s.c. injection, peak radioactivity being detected in the blood liver and spleen after 1 and 24 hr, respectively. This difference most probably reflects a better vascularization of the tumor bed compared with normal tissue.

The persistence of *C. parvum* at the tumor site after intralesional injection may be a factor contributing to the efficacy of this form of *C. parvum* therapy against solid tumors (13, 19, 26). Recent studies by Tuttle and North (29) indicate that a major component of the mechanism of antitumor activity of intralesional *C. parvum* is dependent on the generation of systemic immunity to *C. parvum* antigens which allows an immune-mediated inflammatory response to be focused at the site of *C. parvum* antigens (*i.e.,* the tumor site). This local inflammation was shown to exert a powerful antitumor effect. The prolonged presence of intact *C. parvum* (*C. parvum* antigen) after intralesional injection would be expected to prolong such local reactivity within the tumor site.

Table 3

The relative in vivo persistence of active and inactive strains of ^{125}I-labeled C. parvum during 24 hr after i.v. injection

	Liver		Spleen	
	Active CP[a]	Inactive CP	Active CP	Inactive CP
1 hr	62.3 ± 3.8[b]	40.0 ± 1.6	2.8 ± 0.02	3.7 ± 0.2
24 hr	40.2 ± 3.2	7.5 ± 0.4	1.1 ± 0.02	0.43 ± 0.02
% radioactivity lost during 24 hr	35.5	81.2	60.7	88.4

[a] CP, *C. parvum*.
[b] cpm/organ as a percentage of radioactivity injected.

The *in vivo* antitumor effects of systemic *C. parvum* are considered to be predominantly nonspecific and mediated by activated macrophages (reviewed in Ref. 23). Recent studies on the mechanism of macrophage activation by *C. parvum* implied that 2 activation pathways may operate *in vivo:* direct activation after the ingestion of *C. parvum* by the macrophage, and an indirect activation resulting from the interaction of *C. parvum*-sensitized lymphocytes with *C. parvum* antigen (6, 9). The present finding that peritoneal macrophages are fully activated 4 days after i.v. *C. parvum* treatment in the total absence of *C. parvum*-containing cells in the peritoneum supports the viewpoint that uptake of *C. parvum* by a macrophage is not a prerequisite for its activation. The data do not exclude the possibility that peritoneal macrophages expressing *in vitro* antitumor activity may be the progeny of cells that had taken up *C. parvum* elsewhere and digested it before migrating into the peritoneal cavity. It is likely that the source of such cells may be the bone marrow, since we have shown here that *C. parvum* is taken up by bone marrow cells, and others have shown that *C. parvum* stimulates macrophage production in the bone marrow (11, 30).

Mouse solid tumors have been shown to contain varying numbers of macrophages (10), and the amount of *C. parvum* that localizes within a solid tumor after i.v. injection is most probably a function of the number of these tumor macrophages and the degree of vascularization of the tumor. Overall, the strongest antitumor effects against solid tumors have been achieved by injecting the *C. parvum* directly in the tumor lesion (13, 19, 23, 26), and, on finding that some i.v. injected *C. parvum* localized within a solid tumor, it was considered that a component of the effects of i.v. *C. parvum* may be analogous to an intralesionsl injection. However, the amounts of i.v.-injected *C. parvum* that localized within different solid tumors did not correlate with their sensitivities to i.v. *C. parvum* therapy. It would then seem that the amount of intratumor *C. parvum* found after i.v. injection does not make a critical or significant contribution to the overall antitumor effects of i.v. *C. parvum* therapy. A relevant consideration is our finding that *C. parvum*-activated macrophages, which have been considered to be the predominant effector cell after systemic *C. parvum* (reviewed in Ref. 23), need not contain *C. parvum*.

The *in vivo* degradation of killed *C. parvum* after both i.v. and s.c. injection is a gradual process, and intact FITC-CP were found consistently 2 weeks and more after injection. Interestingly, this resistance to digestion mirrors the chronic stimulation of hyperplasia in the spleen and liver which is detectable 2 to 3 days after i.v. *C. parvum* treatment and gradually increases, peaking around 14 to 16 days (1). Persistence of *C. parvum* has also been noted by Tuttle and North (29), who reported that 6 days after local *C. parvum* injection phagocytic cells at the injection site were still replete with intact bacteria. The data presented here show that "inactive" *C. parvum* (CN5888) was digested far more rapidly than "active" *C. parvum*. Rapid degradation of other inactive bacteria have been reported. Killed ^{131}I-labeled *Salmonella enteritidis* were completely eliminated from the mouse liver within 24 hr of i.v. injection (4). Roberts (21) found that most of the heat-killed *S. typhi* phagocytosed by rabbit macrophages were completely digested within 6 hr, and Topley (28) showed that within 24 hr of being taken up by rabbit splenic histiocytes, *S. paratyphi* N was rendered nonantigenic. In contrast to these is a study with a yeast cell wall extract, zymosan, which is also a reticuloendothelial stimulant (2) with antitumor activity (17). [^{14}C]Zymosan injected i.v. into mice and taken up by the liver persisted in relatively high amounts for a period of several weeks (22). Considering these points, it is interesting to speculate that the relative resistance of naturally occurring reticuloendothelial stimulants to intracellular degradation may contribute to their antitumor activity, and this is now under experimental consideration.

ACKNOWLEDGMENTS

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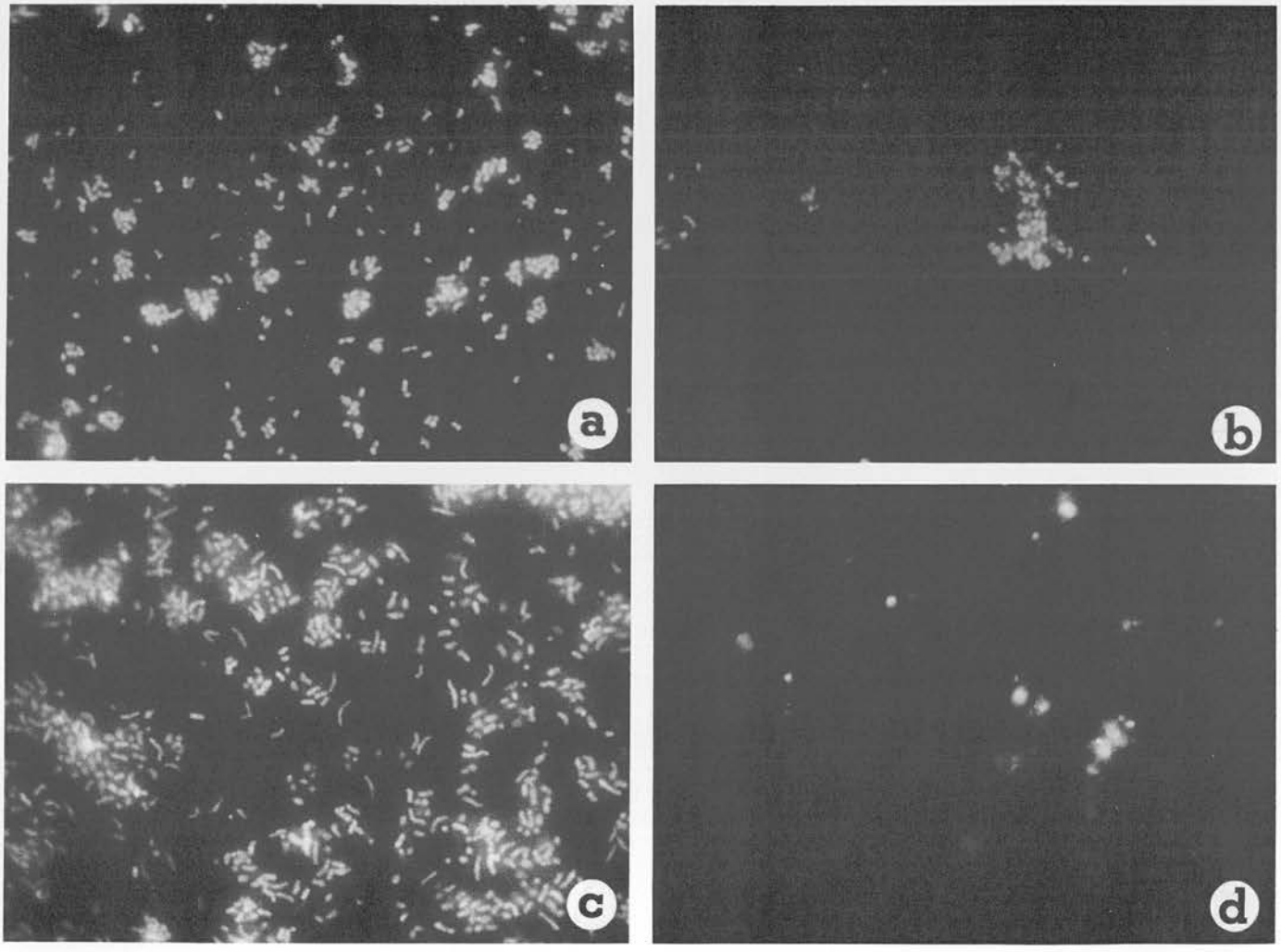

Fig. 1. Fluorescence photomicrographs. *a*, FITC-CP smear; *b*, FITC-CP in spleen section 1 day after i.v. injection; *c*, FITC-CP (CN 5888) smear; *d*, FITC-CP (CN 5888) in spleen section 1 day after i.v. injection. × 1000.

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STUDIES USING LABELLED *C. PARVUM* PREPARATIONS IN MICE

M. T. Scott

ABSTRACT

The *in vivo* distribution of ^{125}I and fluorescein labelled *C. parvum*, after local and systemic injection mice has been studied. After i. v. injection large amounts were found in the liver, spleen and lungs, with less in the lymph nodes. The distribution after s. c. injection was more restricted; the bulk of inoculum was retained at the injection site and draining lymph node, and only small amounts were found in the liver and spleen. *C. parvum* was found in bone marrow, but not peritoneal cells after i. v. injection. It was, however, possible to recruit *C. parvum*-containing cells into the peritoneal cavity by i. p. injection of thioglycollate, and to a lesser extent, i. p. injection of syngeneic tumour cells.

The present studies using labelled *C. parvum* preparations were undertaken to determine its *in vivo* distribution after systemic or local injection and provide further information as to its mode of antitumour action.

Fluorescein and ^{125}Iodine labelled C. parvum *(CP)*

Fluorescent preparations of CP were prepared by mixing CP vaccine (Wellcome) with fluorescein isothiocyanate (FITC) as detailed in (6). At various times after injection of FITC-CP into mice, organs were removed, cryostat sectioned, and examined using phase contrast-fluorescence microscopy. CP was labelled with ^{125}I using a chloramine-T conjugation method described in (6). After injection of ^{125}I-CP organs were removed and counted in an automatic γ counter. Both FITC-CP and ^{125}I-CP retained lymphoreticular stimulatory and antitumour activity (6).

In vivo *distribution of FITC-CP*

300 μg of FITC-CP were injected i. v. into normal CBA mice and 1 and 24 h later various organs, and peritoneal and bone marrow cells were examined for FITC-CP (Table I). After 1 h large amounts were present in the liver, spleen and lungs, with less in the kidneys. Some, but only small amounts, were detected in all lymph node sections (popliteal, inguinal) but only a few organisms were found in occasional thymus sections. Brain sections were negative. All organs were similarly labelled after 24 h except for a drastic loss of FITC-CP from the lungs. Some bone marrow cells contained FITC-CP but none was found in peritoneal cells after i. v. injection (Table I).

Table I. Distribution of FITC-*C. parvum* following i. v. injection. †Semi-quantitative estimate of numbers of FITC-*C. parvum*/unit section. *% cells containing FITC-*C. parvum*

	1 hour	24 hours
Spleen	++++†	++++
Liver	++++	++++
Lungs	++++	+
Node	+	+
Thymus	±	±
Kidney	++	++
Brain	-	-
Bone marrow	10.3 1.2*	7.1 0.3*
Peritoneal cells	-	-

Recruitment of FITC-CP containing cells into the peritoneal cavity

Mice were injected i. v. with FITC-CP and 4 days later (when no FITC-CP containing cells were detectable in either the peritoneal cavity or blood) were given 0.5 ml thioglycollate i. p. (Difco Laboratories, Inc., Detroit, Michigan, U.S.A.). Peritoneal cells were subsequently screened for FITC-CP containing cells (Fig. 1) and these appeared within 1 day and persisted throughout the next 4 days' studies. To determine whether an i. p. injection of syngeneic tumour cells would similarly recruit FITC-CP containing cells, mice were injected i. v. with FITC-CP and 3 days later 10^5 syngeneic R1 leukemia cells (4) were injected i. p. Control mice received only i. v. FITC-CP and 2 days later peritoneal cell samples were screened for FITC-CP containing cells. None was found in control mice, but, following i. p. R1, $0.64 \pm 0.1\%$ of peritoneal cells were positive.

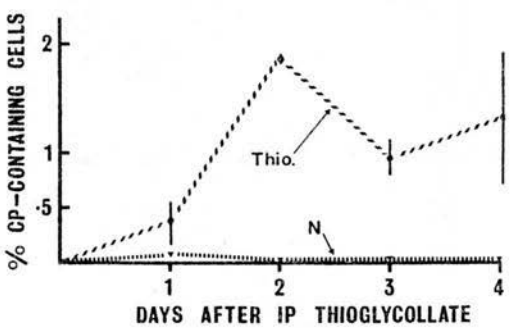

Fig. 1. Recruitment of FITC-CP containing cells into the peritoneum by thioglycollate. Mice were injected i. v. with FITC-CP and 4 days later were injected i. p. with 0.5 ml thioglycollate (Thio.) or were untreated (N). The percentage of peritoneal cells containing FITC-CP at various times after thioglycollate was assayed.

Correlation of FITC-CP with ^{125}I-CP

Microscopic detection of FITC-CP provides unequivocal evidence of the presence of CP organisms, but the results are difficult to quantitate. To determine whether the amount of radioactivity detected in ^{125}I-CP experiments correlated with the presence of FITC-CP detected by fluorescence microscopy, advantage was taken of the rapid loss of CP from the lungs described above. Mice were injected i. v. with 300 μg of either ^{125}I-CP or FITC-CP and the lungs removed 1, 24 and 48 h later. The number of FITC-CP per unit area (20 microscope fields) of lung sections was counted. ^{125}I-CP containing lungs were weighed and the cpm/unit weight of lung determined. The 1 h results were assigned the value of 100% and the result plotted as % CP/unit lung (Fig. 2). The correlation between the loss of FITC-CP and ^{125}I-CP suggests that the amount of radioactivity is representative of the amount of CP present.

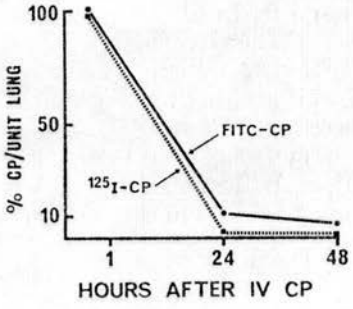

Fig. 2. Correlation of the amounts of ^{125}I-CP and FITC-CP present in the lung at various times after i. v. injection.

Distribution of ^{125}I-CP injected either i. v. or s. c. into normal mice

^{125}I-CP was injected either i. v. or s. c. into a hind footpad. Twenty-four hr later organs were removed for γ counting (Fig. 3). In keeping with the FITC-CP data (Table I) the highest activity was found in the liver and spleen, with intermediate amounts in the lungs, and only small amounts in lymph nodes (popliteal). After s. c. injection the bulk of the inoculum was retained at the injection site. The direct draining node (popliteal) was heavily labelled with

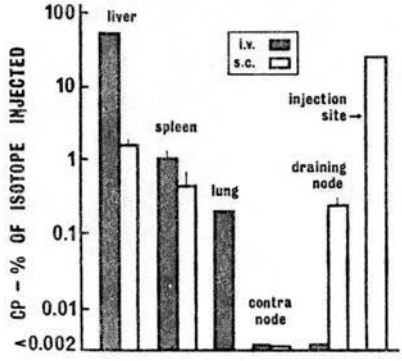

Fig. 3. Relative amounts of ^{125}I-CP in various organs 24 h after i. v. or s. c. (hind footpad) injection (300 μg) into normal CBA mice. The lymph nodes assayed were popliteal; after s. c. injection these were either draining or contralateral (contra) to the injection site.

successively distal nodes (lumbar, inguinal) less so (data not included in Fig. 3). Contralateral nodes were unlabelled and uptake in the liver and spleen was considerably less than after i. v. injection.

DISCUSSION

The presence of CP in the various tissues corresponds with reported biological modifications in them following injection of CP: hepatomegaly, splenomegaly and increased lung weight (1) increased lymph node weight (2), increased lymph node mitogen (3) and antigen (5) responsiveness, decreased thymus mass (2) and increased bone marrow colony forming capacity (8).

That the degree of stimulation caused by CP may be directly proportional to the amount of CP reaching the tissues is suggested by the fact that the restricted distribution of CP after s. c. injection causes intense stimulation of the draining lymph nodes with only minimal splenomegaly and hepatomegaly compared with i. v. CP.

The recruitment of cells containing intact CP into the peritoneum following thioglycollate and, to a lesser extent, tumour cell injection is interesting in that it suggests that a tumour, or tumour associated inflammation may serve to focus CP antigen at a tumour site, thereby initiating, or intensifying immune mediated local anti-tumour activity (7).

Both FITC-CP and ^{125}I-CP have proved useful reagents in further studies using tumour bearing animals and these data are reported elsewhere (6).

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General discussion

Chare: What is the distribution of labelled organisms which possess no anti-tumour activity?

Scott: The organ distribution of labelled preparations of an active strain of *C. parvum* (one that does not have lymphoreticular or anti-tumour activity) is similar to the active strain. The persistence is much less, nearly all of the label having disappeared in 24 hours.

Dimitrov: There is a substantial difference in the residual activity in the kidney when i. v. and subcutaneous routes of administration of the radioactive vaccine are compared. It appears that the radioactivity persists longer after subcutaneous administration.

Scott: I have not looked at persistence of labelled *C. parvum* in the kidney after subcutaneous administration.

Gauthier-Rahman: I should like to make a comment. You found that the peritoneal macrophages had very little *C. parvum* after i. v. administration to rats. But at the times you looked at them, at 1 and 24 hours, you took the resident macrophages.

Scott: We have looked right through to 21 days after administration and we never find any *C. parvum* in the peritoneum.

Gauthier-Rahman: The resident macrophages are not provoked macrophages? Macrophages activated by *C. parvum* are largely of bone-marrow origin and could be studied with a provoked peritoneal exudate.

Scott: In mice, pre-treated i. v. with labelled *C. parvum*, we are able to recruit cells containing *C. parvum* into the peritoneal cavity by a subsequent intra-peritoneal injection of thioglycollate. These may well be of bone-marrow origin.

Cancer Immunol. Immunother. 5, 85–91 (1978)

Cancer Immunology and Immunotherapy

The Distribution and Effects of Intrapleural *Corynebacterium parvum* in Mice

A Comparison with Intravenous *Corynebacterium parvum*

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Summary. *After intrapleural (IPl) injection of* ^{125}I *or fluorescein labelled* C. parvum, *most was confined to the pleural and mediastinal spaces. The pleural phagocytes and mediastinal lymph nodes were heavily labelled, but very little was found in the lung. The amounts of* C. parvum *taken up by the liver and spleen were less than after IV injection and splenomegaly was also less after IPl than IV injection. A large proportion (> 90%) of cells in pleural washouts following IPl* C. parvum *was activated macrophages which inhibited, nonspecifically, the growth of tumor cells in vitro. No similar activity was detected after IV* C. parvum. *IPl injection of* C. parvum *mixed with irradiated tumor cells conferred strong, specific systemic immunity against tumor challenge, and this immunity was also demonstrable using mediastinal lymph node cells in a Winn assay. The immunity resulting from IV* C. parvum *and IPl irradiated tumor cells was significantly lower.*

IPl C. parvum *has been compared with IV* C. parvum *for its effect against tumors growing either in the lung or pleural cavity. Tumors growing in the pleural cavity were inhibited more effectively by IPl than IV* C. parvum. *With tumors growing in the lung (caused by tumor cells injected IV), although IV* C. parvum *was more effective at reducing the number of lung nodules during the first two weeks, the mice consistently survived longer after IPl* C. parvum.

Introduction

McKneally et al. (1976, 1977) have reported encouraging data concerning the treatment of stage I lung cancer patients with post resection injection of BCG into the pleural space. It was apparent from animal studies that the mechanisms underlying the antitumor activity of *C. parvum* were similar to those of BCG (Scott and Bomford, 1976) and in 1977 the Ludwig Lung Cancer Group started a multicentre clinical trial using post resection intrapleural (IPl) *C. parvum* (Ludwig, 1978). The animal studies described here were undertaken in conjunction with this trial with the purposes of defining the distribution of *C. parvum* following IPl injection, the antitumor mechanism(s) engaged, and any effects on experimental lung-associated tumors. These effects have been compared with those resulting from *C. parvum* in-

[1] M.T.S. is a member of the Ludwig Lung Cancer Study Group. The present work arose out of discussions with other members of the group and is presented on their behalf. The study group is: M. Kaufmann, J. Stjernswärd (Ludwig Institut for Cancer Research, Lausanne Branch, Switzerland), M. Zelen, K. Stanley (Frontier Science and Technology Research Foundation, Inc. Amherst, New York, USA), D. S. Freestone, R. Bomford, M. T. Scott, T. Priestman (The Wellcome Research Laboratories, Beckenham, England), C. Mouritzen, G. Ahlbom (Dept. of Thoracic and Cardiovascular Surgery, Aarhus Kommunehospital, Aarhus, Denmark), N. Konietzko, D. Greschuchna (Ruhrland Klinik, Essen-Haidhausen, Germany), P. Hilgard (Innere Klinik und Poliklinik [Tumorforschung] Essen, Germany), J. Vogt-Moykopf, D. Zeidler, H. Toomes (Thoraxchirurgische Spezial-Klinik, Heidelberg-Rohrbach, Germany), F. Krause, R. Rios (Thoraxchirurgische Abt., Fachkrankenhaus für Lungen- und Bronchialerkrankungen, Löwenstein, Germany), J. Orel, M. Benedik, B. Hrabar (Clinical Center, Dept. of Thoracic Surgery, Ljubljana, Yugoslavia), S. Plesnicar (The Institute of Oncology, Ljubljana, Yugoslavia), H. A. Rostad, J. R. Vale (Rikshospital, Oslo, Norway), S. Hagen, S. Birkeland, (Ulleval Hospital, Oslo, Norway), T. Harbitz, R. Nissen-Meyer (Aker Hospital, Oslo, Norway), L. Rodriguez, V. O. Björk, K. Böök (Karolinska Sjukhuset, Thoracic Clinic, Stockholm, Sweden), E. Gradel, J. Hasse, P. Holbro (Kantonsspital,Thoraxchirurgische Klinik, Basel, Switzerland), L. Eckmann (Tiefenauspital, Chir. Univ.-Klinik, Bern, Switzerland), B. Nachbur, T. Liechti (Inselspital, Dept. of Thoracic and Cardiovascular Surgery, Bern, Switzerland), H. Cottier (Inst. of Pathology, Inselspital, Bern, Switzerland), W. Maurer, M. Kaufmann, P. Froelicher (Bürgerspital, Surgical Dept., Solothurn, Switzerland), H. Denck, N. Pridun (Krankenhaus der Stadt Wien-Lainz, Chir. Abt.,Vienna, Austria), K. Karrer (Institute for Cancer Research, University of Vienna, Austria)

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jected IV since this was considered by the Group as a future additional, or alternative, treatment.

Materials and Methods

Mice. Adult CBA/H mice were used throughout.

C. parvum. A killed suspension of *C. parvum* (Wellcome strain CN6134) was provided by Wellcome Reagents Ltd., Beckenham, Kent, England. Dilutions were made with saline.

^{125}Iodine and Fluorescein Isothiocyanate (FITC) Labelled C. parvum. The preparation and characteristics of these reagents, and also the fluorescence microscopy and γ-counting techniques for monitoring their in vivo distribution have been described previously (Scott and Milas, 1977).

Intrapleural (IPl) Injection Technique. This was based on that described by Chu and Lin (1976). Mice were anesthetized with Avertin (Winthrop Laboratories, Surbiton-upon-Thames, Surrey, England), and a small incision made in the left lateral side of the chest; 0.1 ml volumes were injected carefully through an intercostal space using a 27 gauge needle. The incision was closed with autoclips (Clay Adams, Parsippany, New Jersey, USA).

Collection of Pleural Cells. Mice were killed by CO_2 asphyxiation and a ventral mid-line incision made over the chest. Dulbecco's medium (20 ml) containing 5% heparin/ml was injected with a 27 gauge needle into the pleural cavity. The chest wall was gently massaged for a few minutes. A small vertical incision was made with fine pointed scissors and the fluid collected using a Pasteur pipette.

Tumors. The origins, characteristics and maintenance of syngeneic CBA M4 fibrosarcoma (Bomford, 1975), T_3 fibrosarcoma (Bomford and Olivotto, 1974) and RI leukemia (Olivotto and Bomford, 1974) have been described previously.

Tumor Cell Irradiation. M4 fibrosarcoma cells were exposed to 10,000 rads from a ^{137}Cs source.

In vivo Tumor Measurement. M4 fibrosarcoma cells were injected SC (0.05 ml) into a hind footpad, where they grew as a solid tumor. Tumor growth was measured as footpad thickness using a dial gauge caliper.

In vitro Assay for Nonspecific Tumor Inhibition. This technique has been described before (Scott, 1974). Washed peritoneal or pleural cells were mixed in vitro at a ratio of 10 : 1 with RI leukemia cells. ^{3}H thymidine was added after 24 h of culture, and its uptake by RI cells assayed 24 h later. Neither normal nor *C. parvum* stimulated pleural or peritoneal cells incorporated significant amounts of thymidine when cultured alone and the results are expressed as per cent tumor inhibition, calculated as follows:

$$\frac{\text{cpm/culture RI alone} - \text{cpm/culture RI+effector cells}}{\text{cpm/culture RI alone}} \times 100 .$$

Lung Nodule Inhibition Assay. The number of nodules present in the lungs of mice at various times after IV injection of live T_3 fibrosarcoma cells was assayed as described by Bomford and Olivotto (1974).

Winn Assay for Tumor Inhibition. Washed lymph node cells were mixed (10 : 1) with live M4 fibrosarcoma cells. The mixtures were injected SC into a hind footpad of mice which had received sublethal whole body irradiation (500 rads) 1 day earlier. The growth of the tumor in the footpad was then monitored.

Statistics. Results were compared using a Student *t*-test and differences where $P < 0.05$ were taken as significant.

Results

Distribution of Labelled C. parvum *Preparations Following IPl or IV Injection*

In mice the right and left pleural cavities are continuous (unlike in humans where they are separate), and FITC-labelled *C. parvum* injected into one side appeared immediately in samples taken from the other side. The uptake of FITC-labelled *C. parvum* by pleural, peritoneal and bone marrow cells after IPl or IV injection is shown in Table 1. One hour after IPl injection there were still large amounts of *C. parvum* free in the pleural cavity. After 24 h these had been completely phagocytosed and many pleural cells contained *C. parvum*. In contrast, very few *C. parvum*-containing cells were found after IV injection. The uptake of *C. parvum* by bone marrow cells following IPl injection was much less than after IV injection, and no uptake by peritoneal cells occurred after either IPl or IV injection.

Previous studies using labelled *C. parvum* preparations have shown that after 24 h all *C. parvum* has been removed from the blood (Scott and Milas, 1977). Table 2 shows the amounts of radioactivity associated with various organs 24 h after either IV or IPl injection of ^{125}I-labelled *C. parvum*. The relative amounts found after IV injection accord with those described previously (Scott and Milas, 1977). For the spleen and liver these were significantly larger than after IPl injection, whereas uptake by the lungs and mediastinal lymph nodes was larger after IPl injection. Axillary lymph nodes were similarly labelled after either IV or IPl injection.

To confirm the presence of morphologically recognizable *C. parvum* in the various tissues, the experiments were repeated substituting FITC-labelled for ^{125}I-la-

Table 1. Uptake of FITC-*C. parvum* by pleural, peritoneal and bone marrow cells after either IPl or IV injection

Cells	1 h		1 day	
	IPl	IV	IPl	IV
Pleural	free orgs	< 0.5[a]	54.8 ± 5.8	< 0.5
Peritoneal	0.0	0.0	0.0	0.0
Bone marrow	< 0.5	10.3 ± 1.2	< 0.5	7.1 ± 0.3

[a] % cells containing FITC-*C. parvum*

Table 2. Organ distribution of ^{125}I-*C. parvum* 24 h after IV or IPl injection

Injection route	Liver	Spleen	Lung	Axillary node	Mediastinal node
IV	35.0[a] (8.4)	1.1 (0.2)	0.2 (0.002)	0.003 (0.0003)	0.005 (0.001)
IPl	7.2 (2.0)	0.4 (0.3)	6.3 (3.1)	0.006 (0.001)	0.8 (0.3)

[a] Radioactivity – cpm % injected (standard error)

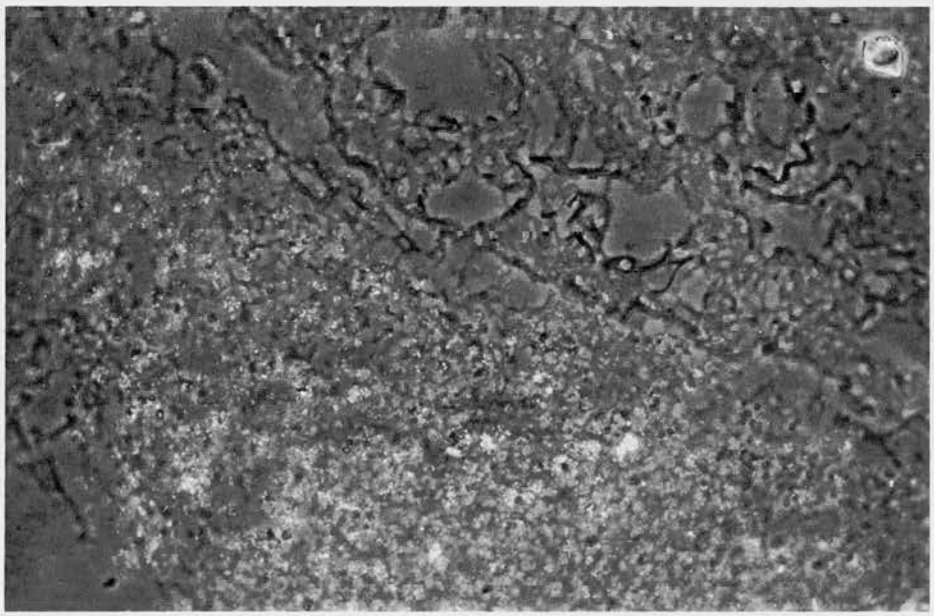

Fig. 1. FITC-labelled *C. parvum* 24 h after IPl injection. No fluorescent organism are within the lung tissue (upper right); they are restricted to cellular aggregates adhering to the lung surface (lower left)

belled *C. parvum* and examining cryostat tissue sections. In all cases, except for the lungs after IPl injection, the fluorescence data confirmed the presence of intact *C. parvum* directly within the tissues and also their relative distribution. After IPl injection only occasional *C. parvum* organisms were seen within the lung tissue but large amounts were contained in aggregates of pleural cells closely associated with the lung surface (Fig. 1). It is known that after IV injection of FITC-labelled *C. parvum* the largest amounts are found in the lung after only 1 h (Scott and Milas, 1977). Further studies in the present series have, however, failed to show significant amounts of *C. parvum* actually within the lung from 1 h up to 7 days after IPl injection.

Splenomegaly After IV or IPl C. parvum

Splenomegaly is a convenient measure of the degree of systemic stimulation resulting from *C. parvum* injection (Adlam and Scott, 1973) which has correlated with antitumor effects (McBride et al., 1975; Riveros-Moreno et al., 1978). Mice were injected either IV or IPl with 350 μg *C. parvum;* 14 days later their spleens were removed and weighed. Control spleen from uninjected mice weighed 0.08 ± 0.02 (SE) g, and those from IV and IPl *C. parvum* injected mice 0.55 ± 0.03 and 0.20 ± 0.08 g respectively ($P < 0.005$).

Nonspecific, in vitro Antitumor Activity of Pleural and Peritoneal Cells After IV or IPl C. parvum

Mice were injected either IV or IPl with 350 μg *C. parvum* and 4 days later their peritoneal and pleural cells assayed for in vitro tumor inhibitory activity against syngeneic target RI leukemic cells. These cells are susceptible to in vitro inhibition by activated macrophages (Olivotto and Bomford, 1974). Both pleural and peritoneal cells from untreated mice were either without effect, or slightly promoted target cell thymidine uptake (Table 3). After IV *C. parvum,* inhibitory activity was restricted to the peritoneal cells. After IPl *C. parvum,* both pleural and peritoneal cells were significantly inhibitory but the activity of peritoneal cells was considerably less than that of pleural cells. The activity of pleural cells

Table 3. Nonspecific, in vitro, tumor inhibitory activity of pleural and peritoneal cells 4 days after either IV or IPl *C. parvum*

C. parvum	Effector cells	% Tumor inhibition[a] ± SE
None	Pleural	− 7.1 ± 1.10
	Peritoneal	− 4.9 ± 1.8
350 μg IPl	Pleural	75.0 ± 2.4
	Pleural (1000 rads)[b]	73.4 ± 0.6
	Peritoneal	22.7 ± 3.5
350 μg IV	Pleural	1.93 ± 0.9
	Peritoneal	82.8 ± 1.3

[a] See Materials and Methods for assay details
[b] Mice irradiated (1000 rads) immediately before removal of cells

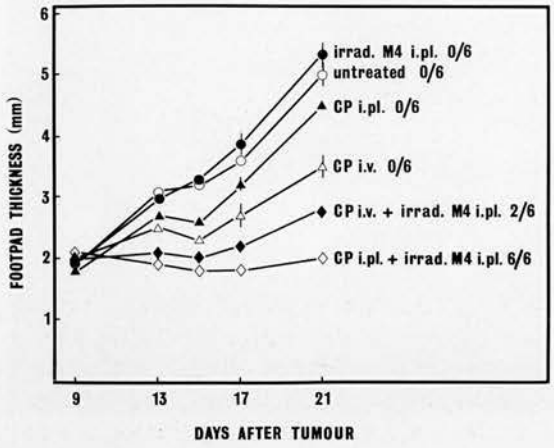

Fig. 2. Groups of 12 mice were injected with 100 μg *C. parvum* (CP) either IV, IPl, or were untreated. Immediately afterwards six mice from each group received 10^6 irradiated M4 tumor cells (irrad. M4) IPl; the remainder were untreated. Seven days later all mice were challenged in a hind footpad with 10^6 live M4 cells and growth of tumor in the foot is shown. Numbers represent the proportion of mice tumor-free at the end of the experiment

stimulated by IPl *C. parvum* was resistant to 1000 rads irradiation delivered to the mice immediately prior to their sacrifice for cell harvest. Pleural cell yields were consistently increased 2–3 fold following IPl *C. parvum* and were composed of > 90% activated macrophages as characterized previously (Bomford and Olivotto, 1974).

Potentiation of Tumor Specific Immunity Following Either IV or IPl C. parvum

Mice were pretreated with either IV or IPl *C. parvum* in combination with IPl irradiated M4 cells. Seven days later they were challenged in a hind footpad with 10^6 live M4 cells and the growth of this tumor was monitored

Table 4. Tumor immunity (Winn assay) expressed in the mediastinal lymph node 6 days after intrapleural injection of *C. parvum*-irradiated tumor cell mixtures

Immunization	Effector cells	Proportion of mice developing tumors in Winn assay
IPl CP[a] + irrad. M4[b]	Med. lymph node	0/5
IPl CP	Med. lymph node	5/5
IPl irrad. M4	Med. lymph node	5/5
None	Med. lymph node	5/5

[a] CP: 100 μg *C. parvum*
[b] Irrad. M4: 10^6 irradiated M4 fibrosarcoma cells

(Fig. 2). Control pretreatments were IV or IPl *C. parvum*, or irradiated M4 cells alone. The IPl injection of irradiated M4 cells alone did not modify growth of the challenge tumor compared with untreated controls. Inhibition of tumor growth after IPl *C. parvum* was significant after 13 days but less than that observed after IV *C. parvum*. The combination of IPl irradiated M4 with IV *C. parvum* was more inhibitory than IV *C. parvum* alone, but the strongest inhibition (all mice tumor free) was achieved when both *C. parvum* and irradiated M4 were injected IPl. The specificity of this immunity resulting from IPl *C. parvum*-irradiated M4 mixtures was demonstrated in further experiments where mice received IPl *C. parvum* mixed with either irradiated M4 cells, or irradiated, antigenically unrelated, RI leukemia cells. Only the latter combination provided significant protection against subsequent live RI cell challenge.

Cell-mediated Expression of Specific Tumor Immunity Following IPl C. parvum

Groups of mice were untreated or injected IPl with 100 μg *C. parvum*, 10^6 irradiated M4 tumor cells, or a mixture of *C. parvum* and irradiated M4. Six days later the mediastinal lymph nodes were removed (these were significantly enlarged following injections containing *C. parvum)*, washed cell suspensions prepared and assayed for antitumor activity in a Winn assay (Table 4). Tumor immunity was only expressed in the mediastinal lymph nodes following injection of *C. parvum*-irradiated M4 mixtures.

Experimental Lung-Associated Tumors

T3 fibrosarcoma cells injected IPl into mice grow as solid deposits restricted to the pleural cavity and shortly after signs of respiratory distress are apparent the mice

Table 5. The effect of either IV or IPl *C. parvum* (350 μg) 1 day before IPl injection of 5×10^4 T3 fibrosarcoma cells

Route of *C. parvum* injection	Survival in days (mean ± SE)
IV	14, 14, 14, 15, 15, 15, 15, 15, 15, 16 (14.8 ± 0.2)[a]
IPl	11, 14, 18, 19, 22, 26, 27, 27, 31 (21.7 ± 2.2)[a]
None	11, 11, 11, 12, 12, 12, 12, 12, 13 (11.8 ± 0.2)[a]

[a] Result significantly different from other groups

Table 6. The effect of 350 μg *C. parvum* injected either IPl or IV 1 day before 5×10^4 T3 fibrosarcoma cells IV

C. parvum injection route	No. of lung nodules ± SE		Mean survival time days ± SE	Proportion of mice surviving
	Day + 7	Day + 14		
IV	2.0 ± 1.0	22.6 ± 8.5	19.2 ± 0.9	0/5
IPl	22.6 ± 8.5	56.2 ± 4.9	35.3 ± 4.7	1/5
–	45.0 ± 11	>100	15.0 ± 0.5	0/5

die. Increased numbers of T3 cells in the inocula shorten the survival times for such mice. T3 cells injected IV grow as discrete nodules in the lung, again eventually killing the mice (Bomford and Olivotto, 1974).

The Effects of Either IPl or IV C. parvum *Against Malignant Pleural Deposits*

Groups of mice were either untreated, or injected IPl or IV with 350 μg *C. parvum*. The following day all mice were challenged IPl with 5×10^4 T3 cells and then monitored for survival (Table 5). IPl *C. parvum* prolonged survival significantly more than IV *C. parvum*.

The Effect of Either IPl or IV C. parvum *Against Tumor Nodules Growing on the Lung*

Groups of mice were injected with 350 μg *C. parvum* either IPl or IV, or were untreated. Seven days later 5×10^4 live T3 fibrosarcoma cells were injected IV. Five mice from each group were assayed for lung nodules 7 and 14 days after tumor challenge and the remainder scored for survival (Table 6). At both times lung nodule inhibition after IV *C. parvum* was greater than after IPl *C. parvum*, however, the ultimate survival of IPl-treated mice was significantly greater and included one survivor (experiment terminated at 60 days). Similar significant differences between the effects of IPl and IV *C. parvum* on both nodule inhibition and survival were obtained when *C. parvum* was given either 7 days before, or up to 8 h after IV tumor cells. Neither IPl nor IV *C. parvum* given more than 8 h after tumor cells consistently caused significant nodule inhibition. In all experiments, survivors occurred only after IPl *C. parvum* – accumulated total 26.7 ± 6.7%. Those killed for lung examinations had no characteristic, well defined white nodules but showed occasional diffuse patches of white pathology.

Discussion

These studies, aimed at characterizing the IPl route of *C. parvum* injection in mice, were carried out in conjunction with the ongoing clinical trial of IPl injection of *C. parvum* into man following surgical resection of lung cancer (Ludwig, 1978). This, in turn, was stimulated by the encouraging clinical data concerning the similar IPl injection of BCG into man (McKneally, 1976, 1977).

The local, as opposed to the systemic nature of the IPl *C. parvum* injection, was apparent from the distribution studies. After IPl injection the bulk of the inoculum was restricted to the injection site (within the pleural phagocytes) and the draining mediastinal lymph nodes.

The amounts of *C. parvum* found in the liver, spleen and bone marrow were considerably less than after IV *C. parvum* injection and this overall distribution is similar to that previously described for *C. parvum* injected SC (Scott and Milas, 1977). It is interesting that between 1 h and 7 days after IPl injection very little *C. parvum* was found within the lung, whereas 1 h after IV injection large amounts were present in the lung (Scott and Milas, 1977). This difference may have a bearing on the relative effects of IV and IPl *C. parvum* against lung and pleural tumors as discussed below.

The antitumor effector systems stimulated by *C. parvum* in mice may be either immunologically specific or nonspecific (reviewed by Milas and Scott, 1978; Scott, 1978). The immunologically nonspecific effects (i.e., independent of specific tumor antigens) seem to predominate after systemic *C. parvum* and are mediated by *C. parvum*-activated macrophages. The local interaction of *C. parvum* and tumor antigen (e.g., after intralesional injection of *C. parvum,* or SC injection of *C. parvum*-irradiated tumor cell mixtures) results in potentiated specific T lymphocyte mediated antitumor immunity. It is apparent that both mechanisms may be engaged by IPl *C. parvum:* nonspecific antitumor activity was expressed in vitro by both pleural and peritoneal cells following IPl injection of *C. parvum* into normal mice, and the local interaction of *C. parvum* and tumor antigen in the pleural cavity resulted in strong, specific cell mediated immunity.

Since recurrence in operable lung cancer may be at a distant site, the degree of systemic antitumor activity resulting from IPl injection of *C. parvum* is an important consideration. The systemic nature of the specific immunity arising from *C. parvum*-tumor antigen interaction in the pleural cavity was evident from the resistance of mice to a distant (footpad) tumor cell challenge. The source of tumor antigen in man might be expected to be that remaining within the pleural cavity or draining lymph nodes after either lobectomy or pneumonectomy. Previous animal studies have shown that for specific immunity to result *C. parvum* and tumor antigen do not have to mix directly as long as the lymphoid drainage of both is common (Scott, 1975).

The nonspecific tumor resistance to a footpad tumor challenge following IPl injection of *C. parvum* alone was significantly less than after IV *C. parvum.* The degree of splenomegaly, which has been correlated with the antitumor effects of systemic *C. parvum* (McBride et al., 1975; Riveros-Moreno et al., 1978) was also less after IPl than IV injection. These facts, along with the observation that after IPl *C. parvum* pleural cells showed greater nonspecific antitumor activity in vitro than did peritoneal cells, suggests that the systemic expression of nonspecific tumor immunity may be more restricted after IPl than IV *C. parvum.*

The ability of IPl *C. parvum* to inhibit the growth of solid tumor deposits growing in the pleural cavity in mice accords with similar findings for the rat where IPl *C. parvum* has reduced both solid tumors and pleural effusions (Pimm and Baldwin, 1977). Pleural macrophages were both increased in number and highly activated after IPl *C. parvum* and it is likely that these were responsible for local tumor destruction within the pleural cavity. Pleural macrophage activation after IV *C. parvum* was insignificant and this may be the basis for its inferior effect against pleural tumor. Since IV *C. parvum* does not enter the peritoneal cavity it has been reasoned that peritoneal macrophages become activated as macrophage precursors (Scott and Milas, 1977). Should this be the case then the minimal amount of pleural macrophage activation detected after IV *C. parvum* suggests that these bone marrow precursors may not enter the pleural cavity as readily as they enter the peritoneal cavity.

Tumor deposits growing within the lung were inhibited more strongly by IV than IPl *C. parvum,* as judged by the numbers of lung nodules present up to 14 days after IV tumor cells. These relative effects are in keeping with the relative amounts of *C. parvum* reaching the lung after either IV or IPl injection and suggest that *C. parvum* within the lung may be triggering a local antitumor effect. Despite the early inferiority of IPl *C. parvum,* however, IPl-treated mice survived longer and long term survivors had very few, and nontypical (regressing?) nodules. Assuming that lung tumor growth is ultimately responsible for death in this model (death consistently follows shortly after the onset of signs of respiratory distress), then these results indicate a delayed, but potent, antitumor effect after IPl *C. parvum.* The nature of this effect is not yet understood. However, preliminary results indicate that it may not operate in T cell deprived mice. Thus our working hypothesis is that whereas the early lung nodule inhibitory effects of *C. parvum* seem to be mediated by immunologically nonspecific means (Bomford and Olivotto, 1974), the delayed effect may be the result of specific T cell mediated immunity arising from *C. parvum*-tumor antigen interaction. Such an interaction would be facilitated if the lungs and pleural cavity had a common lymphoid (mediastinal?) drainage.

Whatever mechanism(s) prove to be operating, it may be stated that, in the present CBA T3 fibrosarcoma model, the IPl route of *C. parvum* injection has been consistently more effective than the IV route against both tumors growing in the pleural cavity and within the lung tissue.

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Cancer Immunology and Immunotherapy

Original Articles

Search for the Possible Role of 'Immunotherapy' in Operable Bronchial Non-small Cell Carcinoma (Stage I and II): A Phase I Study with *Corynebacterium parvum* Intrapleurally

The Ludwig Lung Cancer Study Group[1]

Summary. *The possibility of giving* C. parvum *intrapleurally (i.p.) was investigated.* C. parvum *was given postoperatively either i.p. only or i.p. and intravenously (i.v.) simultaneously. The dose varied from 0.1–10 mg i.p. All patients had been operated for a bronchial carcinoma. Results: (1) Subjective complaints of either dyspnoea, thoracic pain, chills or nausea occured in 31 of 63 patients. No clear dose relation was found. A feeling of discomfort and fever could occur for another 3–4 days after the above more acute symptoms had disappeared. (2) Increased fever (0.5° C) occurred in 71% of the patients injected i.p. only. (3) No anaphylactic reaction was observed. (4) Increased total white blood cell counts (< 20%) occurred in 38 patients. The WBC increase was mainly due to higher number of neutrocytes and granulocytes. Total lymphocyte, monocyte, eosinophilic, and basophilic granulocytes values per mm³ circulating blood remained unchanged, except at the dose of 7 mg* C. parvum *i.p. when monocyte values were increased significantly from 576 ± 247 to 1100 ± 578/mm³. (5) Moderate to severe effusions were observed radiologically in three patients after* C. parvum *intrapleurally.*

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Introduction

Corynebacterium parvum (CP) is known to be a potent stimulator of the immune system (Halpern et al., 1963; Scott, 1974; Woodruff and Dunbar, 1973). In view of its inhibitory effect on tumour growth in a variety of animal models (Bomford and Olivetto, 1974; Cheng et al., 1976; Halpern et al., 1966) it has been suggested that CP may be of value in human cancer (Fisher et al., 1976; Hirshaut et al., 1976; Israel, 1973, 1976). In carcinoma of the bronchus the survival is unacceptably low even in operable cases and the addition of radiotherapy and chemotherapy has failed to improve the prognosis. The role of immunotherapy remains to be confirmed but it has been indicated that a relationship exists between histological signs of immunoreactivity in regional lymph nodes and prognosis in squamous cell bronchogenic carcinoma (Kaufmann et al., 1977). This observation is consistent with other indirect data demonstrating a relationship between immuno-competence and prognosis in lung cancer. Some current randomised clinical trials are exploring the role of immunotherapy in lung cancer. In one BCG is given intrapleurally and the results indicate that local recurrence rates may be reduced in patients with early disease (McKneally et al., 1976). Another

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study uses levamisole and has indicated that distant metastases may be affected (Amery, 1976). An effect by BCG subcutaneously both to inoperable lung cancer (Pines, 1976) and operable (Pouillart et al., 1976) has in two studies with a relative small number of patients been claimed to have a therapeutic positive effect, as has CP given subcutaneously to disseminated epidermoid bronchogenic carcinoma (Israel, 1976). In a recent review, however, it was stated that no single trial to date has conclusively demonstrated a positive role for immunotherapy in malignant disease (Mastrangelo et al., 1976).

The purpose of this study was to investigate the feasibility of giving intrapleural CP post-operatively to patients who had a carcinoma of the lung resected. CP was chosen in preference to BCG for the following reasons:

1. CP suspension contains only killed organisms and there is no need for subsequent neutralisation with other agents.
2. CP is stable for at least 1 year and thus more reliably standardised than BCG (an important factor within a multicentre trial).
3. CP is at least as effective as BCG in its tumour inhibiting effect in animal models (Likhite, 1976; Scott and Bomford, 1976).

Materials

Patients. Sixty-three patients were entered into this study from eight of the European clinics within the Ludwig Lung Cancer Study Group. All patients had a bronchogenic carcinoma and the majority of these were Stage I and II (53/63). All but one patient were treated either by lobectomy (45/63) or pneumonectomy (17/63), one patient with mediastinal node involvement had an exploratory thoracotomy only.

C. parvum. CP was supplied by Burroughs Wellcome (Batch No. BA 3974) and consisted of a suspension of washed formalin-killed organisms in physiologic pyrogen-free saline containing 0.01% thiomersal as preservative, each ampoule containing 7 mg dry weight organisms in 1 ml. CP was given either intrapleurally (i.p.) (46 patients) or intrapleurally and intravenously (i.p. and i.v.) simultaneously (17 patients). The i.p. dose of CP varied from 0.1–10 mg, when given i.p. and i.v. a total dose of 7 mg CP was used, 3.5 mg being given by each route. CP was administered following removal of the thoracic drainage tubes between the sixth and tenth post-operative day. When given i.p., CP was diluted in 5–10 ml of physiological saline and administered via a catheter placed at the time of the operation with its tip at the tracheal bifurcation, at the side of the bronchial resection line. The position of the catheter was ensured with a catgut suture. CP was injected slowly through the catheter which was afterwards flushed with an equivalent volume of saline prior to removal. When CP was given i.p. and i.v. for the i.v. route 3.5 mg CP was diluted in 100 ml of physiological saline and was given by i.v. infusion over 1 h.

Local and systemic effects of CP administration were monitored as follows. Total and differential white blood cell counts (WBC) were performed immediately before and on the first, second, and third day after CP injection. The erythrocyte sedimentation rate (ESR) was measured before and 3 days after CP administration. Temperature and blood pressure were recorded every 12 h, respiration and pulse every 4 h, following CP. Chest X-ray was performed 1 or 2 days before and 3 days after CP. Subjective complaints following CP, such as dyspnoea, cheast pain, chills, and nausea, were noted in relation to time of onset and duration.

Results

Table 1 summarises the data on individual patients, listed according to dose of CP given. The individual complications observed, the effect on white blood differentials, erythrocyte sedimentation rate, temperature, pulse rate, blood pressure, are listed.

Subjective Complaints. Subjective complaints of either chest pain, pleuritic pain, chills, and/or fever, occurred in 31 of the 63 patients and 32 patients had no complaints whatsoever (Table 1, last column). No clear relationship between dose of CP and incidence of subjective complaints was observed. Subjective complaints were more frequent among patients receiving CP i.v. and i.p. (Fig. 1).

Other Changes. Moderate to severe pleural effusions were observed in three patients and minor radiological changes were seen in five patients (Table 1). There was no correlation to dose of CP administrated.

Changes in blood pressure showed no correlation with dose. Four patients receiving i.p. CP experienced an increase of systolic blood pressure greater than 30 mm Hg at doses of 2.5, 4.0, 5.0, and 10.0 CP respectively. Three patients receiving i.p. and i.v. CP experienced similar changes. One patient receiving i.v. and i.p. CP experienced severe hypotension (BP 60/50) accompanied by supraventricular tachycardia 60 h after administration. The paresis of the recurrence nerve observed in one patient happened immediately after CP injection. The paresis disappeared gradually and completely (Table 1).

Temperature Changes. An elevation of temperature greater than 0.5° C following CP administration was considered a febrile response. After i.p. CP, 71% of patients had a febrile response (70% of lobectomy patients and 76% of those who had pneumonectomy). After i.v. and i.p. CP, 88% of patients suffered a febrile response and the maximum temperature elevations were recorded in this group (Figs. 2 and 3). The maximum temperature elevation occurred earlier in lobectomy patients compared to pneumonectomy patients (Fig. 2). There was a tendency of increased temperature with an increased

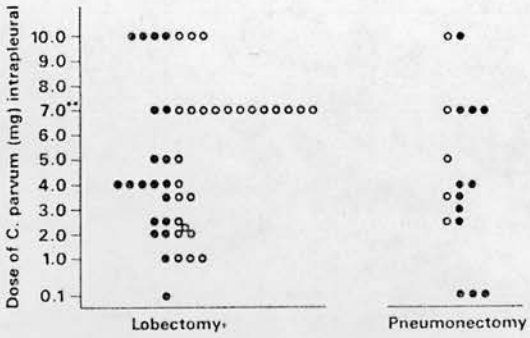

Fig. 1. Subjective complaints (dyspnoea, thoracic pain, chills, nausea) compared to *C. parvum* dosage: ● No complaints, each symbol represents one patient, ✪ Complaints = a patient with any of symptoms listed below; ** All but one patient received the 7 mg dose as 3.5 mg i.v. and 3.5 mg i.p. simultaneously

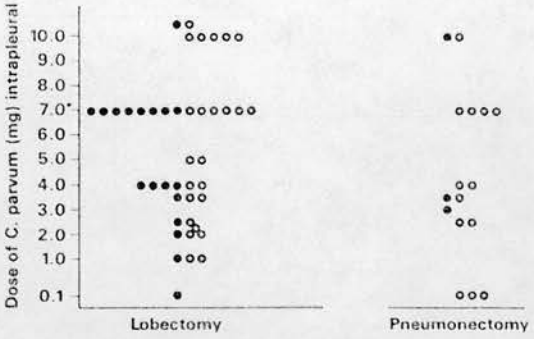

Fig. 3. Maximum temperature in relation to dose of *C. parvum*. Intrapleural *C. parvum:* ○ Lobectomy, △ Pneumonectomy; Intrapleural and intravenous *C. parvum:* ● Lobectomy, ▲ Pneumonectomy; * All but one patient received the 7 mg dose as 3.5 mg i.v. and 3.5 mg i.p.

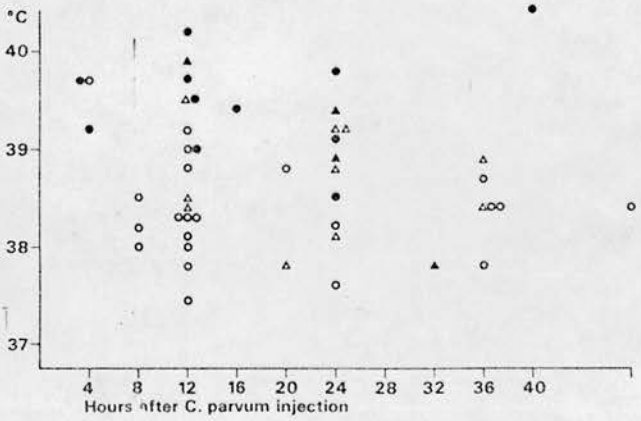

Fig. 2. Maximum temperature after *C. parvum* injection. Intrapleural *C. parvum:* ○ Lobectomy, △ Pneumonectomy; Intrapleural and intravenous *C. parvum:* ● Lobectomy, ▲ Pneumonectomy

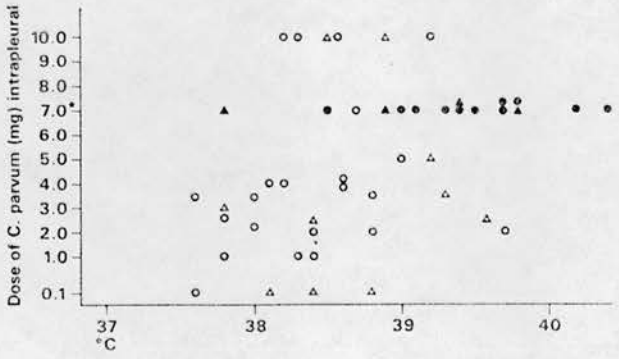

Fig. 4. Increase of white blood cell (WBC) count in relation to dose of *C. parvum:* ● Increase of WBC after *C. parvum* injection ≤ 20%, ✪ Increase of WBC after *C. parvum* injection > 20%; * All but one patient received the 7 mg dose as 3.5 mg i.v. and 3.5 mg i.p. simultaneously

dose of CP, with a clear increase when i.v. and i.p. injections of CP were given simultaneously (Fig. 3).

Changes in Blood Differentials. Venous blood cell differentials were done immediately before CP administration and thereafter during the first, second, and third day. The maximum values which occurred are given in Table 1 and Figure 4.

An increase in the total WBC of more than 20% was observed in 38 patients following CP administration. The increase in WBC is not related to the amount of CP given and is principally accounted for by an increase in the neutrophil compartment although monocytes are also increased at higher doses (Table 2). Table 2 gives a summary of the changes occurring before and after CP injection in relation to dose given. Independently of the dose given, there is after intrapleural CP an increase in the total number of WBC per mm^3 venous blood. Thus a change from 8,940 ± 2,994 to 13,940 ± 6,299 with a dose betwen 0.1 and 2.2 mg and from 9,320 ± 2,776 to 15,520 ± 6,383 when 7–10 mg were given intrapleurally was observed. The increase in total number of WBC is mainly due to a neutrocytosis. There is no change seen for the eosinophiles, basophiles, nor do lymphocytes or monocytes change in total number except for the higher dose of 7–10 mg of CP when a doubling of the monocyte number per mm^3 was observed, from 576 ± 247 to 1100 ± 578.

Table 1. Reactions to *C. parvum* intrapleurally

	Sequence No.	Patients initials	Year of birth	Sex	Clinic (1)	Type of operation	Dosage of C.parvum (mg)	Postop. day of C.parvum injection	Radiological change in pleural effusion after C.parvum	WBC/mm^3 before C.parvum	WBC/mm^3 after C.parvum 3)	Max. percentage increase in total WBC after C.parvum 4)
Ia C. parvum i.p. only Dosage 3.5 mg.	1	M.G.	1906	m	4	P	0.1	6.	+	6600	8500 (3)	29
	2	S.A.	1915	f	4	P	0.1	7.	0	9300	11600 (1)	25
	3	W.E.	1915	m	6	P	0.1	7.	0	9100	18500 (3)	50
	4	H.K.	1917	m	6	L	0.1	10.	0	4900	6400	insig
	5	G.A.	1920	m	6	L	1.0	8.	0	14700	14300 (1)	insig
	6	Z.R.	1926	m	6	L	1.0	7.	0	9600	24700 (3)	156
	7	B.A.	1918	m	3	L	1.0	8.	0			
	8	F.A.	1913	m	3	L	1.0	6.	0	7600	12900 (1)	70
	9	B.U.	1914	m	3	L	2.0	10.	0			
	10	T.S.	1911	m	8	L	2.0	5.	0	5800	6630 (2)	insig
	11	G.H.	1939	m	6	L	2.0	8.	+	12600	22500 (3)	45
	12	P.P.	1917	m	6	L	2.0	7.	0	6800	9200 (1)	30
	13	N.A.	1900	m	3	L	2.2	10.	+++	9200	13400 (1)	46
	14	V.B.	1916		1	L	2.5	7.	0	13900	11800 (2)	insig
	15	J.C.	1919	m	1	P	2.5	8.	0	16800	24000 (1)	44
	16	B.E.	1928	m	2	T	2.5	5	+	7700	10100 (2)	31
	17	J.H.	1926	m	4	P	2.5	6.	0	6200	17500 (3)	182
	18	N.S.	1924	m	8	L	2.5	5.	0	9000	11600 (1)	29
	19	L.R.	1909	m	3	P	3.0	10.	0	12300	12400 (3)	insig
	20	A.O.	1911	m	1	P	3.5	7.	0	15300	18600 (2)	21
	21	A.M.	1911	f	2	L	3.5	6.	++	7700	15700 (1)	104
	22	L.C.	1930	m	3	L	3.5	11.	0	12600	12700 (2)	insig
	23	S.K.	1909	m	3	P	3.5	12.	0	7100	6800 (1)	insig
	24	K.R.	1904	f	7	L	3.5	10	+++	7900	16800 (2)	112
Ib C. parvum i.p. only Dosage 3.5 mg.	25	U.H.	1937	m	1	L	4.0	6.	0	8800	18300 (2)	108
	26	I.D.	1922	f	1	L	4.0	6.	0	10600	10600 (3)	insig
	27	S.W.	1911	m	3	P	4.0	13.	0	12300	15300 (1)	24
	28	H.E.	1925	m	3	P	4.0	11	0	10200	12500 (2)	23
	29	P.J.	1907	m	4	L	4.0	8.	0	8300	10000 (3)	insig
	30	D.C.	1921	f	4	L	4.0	7.	0	5000	7000 (2)	40
	31	H.L.	1916	m	8	L	4.0	4.	0	8900	8600 (3)	insig
	32	S.B.	1918	m	8	L	4.0	6.	0	11700	11700 (1)	insig
	33	S.M.	1931	f	4	L	5.0	8.	0	5400	17600 (1)	226
	34	A.A.	1902	f	4	L	5.0	8.	0	5000	12200 (2)	104
	35	G.J.	1927	f	8	L	5.0	5.	0	7200		
	36	G.S.	1924	f	8	P	5.0	5.	0	9300		
	37	V.D.	1936	m	3	L	7.0	12.	0	5700	16900 (1)	199
	38	G.R.	1917	m	7	P	10.0	12.	0	11200	13900 (2)	insig
	39	H.E.	1918	m	8	L	10.0	15.	0	13300	16100 (2)	21
	40	E.H.	1934	m	8	P	10.0	6.	0	11400	27000 (1)	137
	41	E.A.	1912		8	L	10.0	7.	0	6600	11400 (1)	72
	42	S.F.	1904	m	3	L	10.0	9.	0	107000	17200	61
	43	A.H.	1917	m	3	L	10.0	10.	0	6800	8900 (1)	31
	44	M.A.	1914	m	3	L	10.0	10.	+	12500	24900 (2)	100
	45	R.F.	1906	m	7	L	10.5	14.	0	7200	7900 (1)	insig
	46	V.E.	1905	m	7	L	10.5	14.	0	7800	11000 (1)	41
II C. parvum 3.5 mg i.p. and 3.5 mg i.v.	47	P.A.	1917	m	1	L	3.5 + 3.5	6.	0	7200	7400 (1)	insig
	48	K.O.	1925	m	1	P	3.5 + 3.5	7.	0	8600	30600 (1)	257
	49	H.H.	1906	m	1	L	3.5 + 3.5	10.	0	12600	23900 (1)	100
	50	P.O.	1931	m	1	P	3.5 + 3.5	6.	0	10900	13800 (3)	26
	51	P.A.	1913	f	3	L	3.5 + 3.5	8.	0	18500	21600 (1)	insig
	52	S.C.	1917	m	3	L	3.5 + 3.5	8.	0	7300	12600 (1)	73
	53	J.S.	1914	f	3	L	3.5 + 3.5	7.	0	11100	13300 (1)	insig
	54	Z.E.	1926	m	7	L	3.5 + 3.5	10.	0	11900	13900 (1)	insig
	55	A.R.	1905		8	L	3.5 + 3.5	4.	0	12600	14900 (2)	insig
	56	G.B.	1920		8	P	3.5 + 3.5	5.	0	6900	9700 (1)	40
	57	S.J.	1917	m	8	L	3.5 + 3.5	5.	0	9000	12500 (2)	39
	58	A.K.	1915	m	5	L	3.5 + 3.5	7.	0	11700	9900 (2)	insig
	59	J.T.	1919	m	5	L	3.5 + 3.5	7.	0	7600	11200 (1)	47
	60	S.J.	1914	f	6	L	3.5 + 3.5	8.	0	8300	10000 (3)	21
	61	G.A.	1931	m	6	L	3.5 + 3.5	8.	+	9500	11800 (3)	insig
	62	S.V.	1921	m	6	L	3.5 + 3.5	8.	0	10900	12400 (3)	insig
	63	P.C.	1918	m	1	P	3.5 + 3.5	8.	0	16700	22600 (1)	35

(1) Clinic in which patients were investigated: 1 = Aarhus, 2 = Basel, 3 = Bern (Insel) 4 = Essen, 5 = Ljubljana, 6 = Löwenstein, 7 = Solothurn, 8 = Stockholm.

(2) Radiological change in pleural effusion after C.parvum: 0 = unchanged, + = little ++ moderate, +++ severe.

(3) Day on which maximum change occured is given in parenthesis.

(4) Percentages 20% are indicated by "insig" (insignificant).

(5) Changes of 0.5° are indicated as insignificant.

6) Time interval (hr) C. parvum administration / max. change of temperature, pulse rate, blood pressure respectively is given in parenthesis.

7) Changes of 20 beats/min are indicated as insignificant.

8) Changes in systolic pressure of 30 mmHg are indicated as insignificant.

9) No significant complaints, reactions respectively are indicated by "0".

Discussion

This study was designed as a pilot investigation to explore the feasibility of intrapleural CP administration with a view to carrying out randomised clinical trials using this technique. The results reported indicate that intrapleural CP therapy is possible without undue toxicity, although subjective side effects are increased when CP is given i.v. and i.p. simultaneously.

For future studies we intend to adopt a dose of 7 mg CP i.p. as the standard. This decision is based on the observation that a dose of 7 mg combines a measurable systemic effect (increased neutrophil and monocyte counts) with acceptable toxicity.

The observation that pyrexia is seen earlier in patients after lobectomy than after pneumonectomy may have one of two explanations. Either the greater quantity of pleural fluid post-pneumonectomy has a diluting

Max. temperature after C.parvum 5,6)	Max. pulse rate after C.parvum 6,7)	Max. (min) blood pressure after C.parvum 6,8)	General complaints / reactions 9)
38.1 (24)	insig	insig	0
38.4 (12)	insig	insig	0
38.8 (24)	insig	insig	0
37.6 (12)	insig	insig	0
37.8 (12)	120 (4)	insig	moderate chest pain
38.4 (48)	136 (24)	insig	pleuritic pain; patient died 10 days after C.parvum (cerebral metastasis).
insig	insig	insig	chest pains some hours following injection
38.3 (12)	insig	insig	0
39.7 (4)	insig	insig	chills 4 to 8 hours following C.parvum injection
insig	insig	insig	0
38.4 (12)	120 (4)	insig	moderate dyspnoea, pleuritic pain
38.8 (12)	120 (24)	insig	0
38.0 (8)	insig	insig	chills, dyspnoea, fever, pleural effusion, onset 4 hours post injection
insig	insig	insig	0
39.5 (12)	120 (8)	insig	fever max. 8 hours after injection
insig	120 (16)	insig	thoracic pains 4 hours after injection lasting 2 days
38.4 (36)	128 (32)	180/100 (24)	0
37.8 (36)	insig	insig	0
37.8 (20)	insig	insig	0
39.2 (24)	100 (20)	insig	0
37.6 (24)	100 (36)	insig	severe chest pain
38.8 (20)	120 (8)	insig	0
insig	insig	insig	continuous thoracic pain
38.0 (12)	138 (8)	insig	2 to 4 hours after injection thoracic pain (severe), nausea
38.6 (36)	100 (24)	170/110 (24)	chest pains onset 4 hours following injection
38.6 (36)	108 (36)	insig	0
insig	108 (16)	insig	0
insig	insig	insig	0
insig	insig	insig	0
38.2 (24)	108 (8)	insig	0
insig	insig	insig	0
38.1 (12)	insig	insig	0
insig	insig	insig	0
insig	insig	195/90 (24)	0
39. (12)	insig	insig	chest pain, pleuritic pain, chills and fever
39.2 (24)	insig	insig	pleuritic pain 2 hours after C.parvum injection
38.7 (36)	insig	insig	severe pleuritic pain, dyspnoea a few hours after C.parvum injection
38.5 (12)	152 (28)	insig	0
38.3 (12)	108 (12)	insig	0
38.9 (36)	120 (36)	insig	severe pleuritic pain, left recurrent laryngeal nerve pasly 4 days after C.parvum injection
39.2 (12)	insig	insig	moderate shivering during 1 h, 5 hours post injection
38.5 (8)	insig	insig	dyspnoea shortly after injection lasting 30 minutes
insig	insig	insig	0
38.3 (8)	140 (4)	230/100 (4)	chest pain, loss of appetite, deterioration of patient, high temperature
insig	insig	insig	0
insig	insig	insig	0
39.8 (24)	120 (4)	insig	0
37.8 (32)	132 (4)	insig	0
39.1 (24)	100 (24)	insig	0
38.9 (24)	116 (24)	insig	0
insig	insig	190/100 (8)	chills 8 hours after C.parvum injection
39.2 (4)	100 (4)	insig	severe chills and fever 3 to 4 hours after C.parvum injection
39.7 (4)	insig	insig	chills 2 hours after C.parvum injection.
39.5 (12)	156 (60)	60/50 (60)	chills and fever 4 hr after C.parvum inj. Supraventricular paroxysmal tachycardia 60 hr after C.
38.5 (24)	112 (4)	170/ (4)	slight shivering 30 min after C.parvum injection. / parvum inj. Pulse rate 156, blood pressure
39.8 (12)	114 (32)	insig	fever. / 60/50 at this moment.
39.7 (12)	130 (8)	175/80 (24)	mild shivering 2 hours after C.parvum injection.
insig	106 (4)	insig	0
39.4 (16)	120 (16)	insig	chills, sweating, moderate dyspnoea
39.0 (12)	insig	insig	dyspnoea, pleuritic pain 4 hr after C.parvum injection, increase of pleural effusion
40.4 (36)	120 (12)	insig	dyspnoea, pleuritic pain
40.2 (12)	insig	insig	severe pain, moderate dyspnoea.
39.4 (24)	112 (24)	insig	0

effect on the CP and delays its action or the residual lung tissue present in lobectomy patients leads to a more rapid absorption of CP.

One patient had paroxysmal tachycardia with blood pressure fall to 60/50 mm Hg. It occurred 60 h after CP injection i.p. It is uncertain whether the hypotension observed relates to the CP given earlier. However we have agreed that our patients in the future will have their CP in the morning. Furthermore, the contra-indications for giving CP ought to be respected. They are: patients with any severe allergic disorder or history of endotoxic shock, significant hypertension (e.g., resting diastolic blood pressure of more than 100 mm Hg), current pregnancy, patients with disturbance in blood clothing mechanisms, gross impairment of liver or renal function, long-term steroid administration, primary or secondary cerebral neoplasms, patients with known thiomersal sensitivity, coexisting major untreated infection and per-

Table 2. White blood cells (WBC) and WBC subpopulations before and after *C. parvum*

Dose (mg)	0.1–2.2*		2.5–3.5*		4.0–5.0*		7.0**		7.0–10.5*	
No. of patients	13		11		12		17		10	
C. parvum	Before	After	Before	After	Before	After	Before	After	Before	After
WBC total (X ± SD)	8,940 ± 2,994[a]	13,940 ± 6,299[a]	10,590 ± 3,695	14,363 ± 4,739	8,602 ± 2,717[c]	12,380 ± 3,590[c]	10,881 ± 3,228[e]	13,143 ± 5,898[e]	9,320 ± 2,776[h]	15,520 ± 6,383[h]
Granulocytes										
Unsegmented	230 ± 282	221 ± 381	555 ± 685	737 ± 983	425 ± 533	356 ± 533	246 ± 312	723 ± 1,167	560 ± 377[i]	1,483 ± 1,053[i]
Segmented	5,579 ± 1,992[b]	9,528 ± 4,893[b]	7,034 ± 3,251	10,344 ± 4,699	5,356 ± 2,229[d]	8,847 ± 3,469[d]	7,199 ± 3,255[f]	10,565 ± 5,119[f]	6,319 ± 2,632[j]	11,064 ± 5,049[j]
Eosinophils	207 ± 182	149 ± 106	352 ± 210	230 ± 253	289 ± 173	299 ± 207	419 ± 239[g]	168 ± 165[g]	277 ± 198	233 ± 377
Basophils	39 ± 87	29 ± 50	61 ± 57	29 ± 53	60 ± 56	130 ± 366	41 ± 49	16 ± 32	60 ± 85	64 ± 88
Lymphocytes	1,948 ± 892	2,814 ± 1,536	1,908 ± 794	1,513 ± 505	1,559 ± 583	1,664 ± 646	2,068 ± 1,088	1,793 ± 1,498	1,503 ± 476	1,655 ± 527
Monocytes	529 ± 257	920 ± 615	681 ± 140	730 ± 365	580 ± 260	734 ± 422	527 ± 245	582 ± 396	576 ± 247[k]	1,100 ± 578[k]

* *C. parvum* given intrapleurally

** *C. parvum* given as 3.5 mg intravenous and 3.5 mg intrapleural simultaneously

Statistically the following levels of difference were obtained:

[a] $t = 2.56$ ($P < 0.05$)
[b] $t = 2.34$ ($P < 0.05$)
[c] $t = 2.46$ ($P < 0.05$)
[d] $t = 2.22$ ($P < 0.05$)
[e] $t = 2.09$ ($P < 0.05$)
[f] $t = 1.94$ ($P < 0.05$)
[g] $t = 2.20$ ($P < 0.05$)
[h] $t = 2.81$ ($P < 0.05$)
[i] $t = 2.60$ ($P < 0.05$)
[j] $t = 2.63$ ($P < 0.05$)
[k] $t = 2.63$ ($P < 0.05$)

haps also patients with coexisting auto-immune disorder, e.g., collagen disease.

The search for the possible role of immunotherapy in humans by immunomodulators known to have a tumor-inhibiting effect in experimental animal systems is still in a very crude primary stage. The present study shows how difficult it is to transfer a simple question such as optimal dose from mouse to man and explore a new route of administration. 'Unspecific immunotherapy' in the close vicinity of the tumor at the site for recurrence is proven to be efficient for different human tumours localised in the skin (Stjernswärd and Levin, 1971; Klein and Holterman, 1972) and is more likely to give an effect than e.g. unspecific 'immunotherapy' far away from the areas of tumour recurrence.

The hig . number of patients with bronchogenic carcinoma the early tumour recurrence after 'radical' resection (minimal tumour burden realistic for immunotherapy) and the inefficiency of present treatment modalities such as radiotherapy and chemotherapy (Second Natl. Cancer Inst. Conf. on Lung Cancer, Airlie House, Virginia, USA, in press, 1977) offers a realistic opportunity to analyse conclusively and in a short time whether the proposed immunotherapy could improve prognosis. The i.p. route is an attempt to bring CP in proximity to the micro metastases and for recurrences known to occur intrathoracically in a very high frequency even in operable lung cancer. CP given i.p. certainly also has a systemic effect, as revealed by e.g. increase in total number of circulating WBC.

The present study shows for the first time that it is feasible to give CP intrapleurally and indicates which dose can be used clinically. This is now being done in a clinical trial with 7 mg of CP intrapleurally in the search of the possible role of *C. parvum* 'immunotherapy' postoperatively in operable bronchial carcinoma.

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Antitumor Activity of Purified Cell Walls From *Corynebacterium parvum* [1]

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ABSTRACT—Cell walls (CW), containing peptidoglycan and carbohydrate, were prepared from *Corynebacterium parvum* and tested for lymphoreticular stimulation and antitumor effects in CBA-T6T6 mice. CW did not induce splenomegaly. Peritoneal macrophages became cytostatic to RI leukemia cells in vitro after ip injection of CW or of peptidoglycan but not of carbohydrate; however, on a dry-weight basis the activity was low (<10%) compared with that of *C. parvum*. Tumor outgrowth was significantly suppressed after sc injection of mixtures of M4 fibrosarcoma cells and CW, but again the activity of CW was less than 10% of that of *C. parvum*. In contrast to injection of *C. parvum*, intratumor injection of CW failed to retard tumor growth in normal mice, although a suppressive effect was found in mice presensitized to *C. parvum*. Again, unlike *C. parvum*, CW did not act as an adjuvant for tumor-specific transplantation antigen, as judged by a lack of enhanced resistance to tumor challenge after injection of mixtures of CW and irradiated M4 cells. The distribution and persistence of ^{125}I-labeled *C. parvum* and CW after sc or ip injection were similar. CW activity was not restored by attachment to oil droplets or emulsification in Freund's incomplete adjuvant.—J Natl Cancer Inst 60: 653-658, 1978.

Corynebacterium parvum is a potent stimulator of the lymphoreticular system (*1*), an immunologic adjuvant (*2*), and an antitumor agent (*3*).

The component(s) of *C. parvum* responsible for these effects is not yet known. Bacterial fractions containing CW of *C. parvum* (in some cases, of other anaerobic coryneform bacteria or propionibacteria) retain lymphoreticular stimulatory (*4*), adjuvant (*5–8*), and antitumor properties (*9–11*).

We therefore examined the lymphoreticular stimulatory activity and both nonspecific and specific antitumor activities of purified CW of *C. parvum*. A drastic loss of activity in CW was found, and some possible causes of this were investigated.

MATERIALS AND METHODS

All studies reported here were performed with CW or fractions obtained from chemically untreated, broken bacteria. Cultures of *C. parvum* (Wellcome strain CN 6134) were supplied by the Department of Bacteriology, The Wellcome Research Laboratories. Fresh cultures of bacteria, grown in 1% glucose broth, were centrifuged at 5° C in a Sorvall KSB continuous flow system at 12,000×*g*. The cell pellet was washed similarly with cold saline and stored as a cell paste at −20° C until used.

Preparation of CW.—The bacteria were broken in a Novotny cell disruptor (*12*) with a modified pestle of monocast nylon (Polypenco, CM901; Imperial Chemical Industries Ltd., Millbank, London, England). The cells were resuspended in 0.1 M tris buffer, pH 7.6. Ballotini beads No. 12 were added, and the mixture was prepared at 10,000 rpm for 1½ minutes on ice+NaCl in a Silverson homogenizer (Silverson Machine Ltd., Waterside, Chesham, Bucks, England). The homogenate was filtered through an ice-cold sintered glass funnel, and the glass beads were washed with four times the original volume of the same ice-cold buffer. After spinning at 1,000×*g* for 20 minutes at 4° C in a Sorvall RC5 centrifuge, the pellet contained mainly complete cells and was routinely discarded. The supernatant was treated as detailed in text-figure 1. After SDS, trypsin, DNase, and RNase (B.D.H. Chemical Ltd., Poole, Dorset, England) treatments, the CW were extensively washed and finally freeze-dried.

Analytic methods.—Protein was determined by the method of Lowry et al. (*13*); hexose, by the phenol-H_2SO_4 method of Dubois et al. (*14*); and phosphate, by the method of Ames and Dublin (*15*). Amino acids were determined automatically by use of a Beckman model 120C amino acid analyzer.

Hydrolysis.—For amino acid and amino sugar analyses the CW and fractions were hydrolyzed in 4 N HCl for 4 hours at 108° C in Pierce vacuum hydrolysis tubes (Pierce Chemical Co., Rockford, Ill.). After hydrolysis, samples were dried in vacuo in the presence of P_2O_5 and NaOH pellets. For the analysis of sugars and amino sugars, samples were hydrolyzed in 2 N H_2SO_4 by being heated in a boiling water bath for 2 hours. Neutralization was achieved by the addition of solid $BaCO_3$ and, after centrifugation and washing of the pellet, the combined supernatants were evaporated to dryness under P_2O_5.

Chromatography.—Sugars were analyzed qualitatively by descending chromatography on Whatman #1 paper. The solvent used was ethyl acetate:pyridine:water (8:2:1), and the chromatograms were run for 24 hours at room temperature. Compounds were detected by use of the silver nitrate method of Trevelyan et al. (*16*).

Preparation of CW carbohydrate and peptidoglycan fractions.—Purified CW, as described above, were mildly hydrolyzed in 0.1 M acetic acid for 1 hour in a boiling water bath. The mixture was centrifuged, and the

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ABBREVIATIONS USED: CW = cell wall(s); SDS = sodium dodecyl sulfate; N = normal; DHR = delayed hypersensitivity reaction.

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[4] We acknowledge the skillful technical assistance of Anne Niblock, Susan Wishart, Iain Jaundrell-Thompson, and Anthony Ford.

supernatant containing the carbohydrate was freeze-dried in the presence of solid NaOH. The peptidoglycan-containing pellet was rehydrolyzed and, after centrifugation, similarly freeze-dried.

Preparation of CW associated with oil droplets.—The technique used was exactly as described for BCG by Zbar et al. (*17*), except that CW were substituted for mycobacterial components. Phase contrast microscopic examination of preparations confirmed the association of some of the CW with oil droplets.

Assay for C. parvum *serum agglutinins.*—This test was as described previously (*18*).

Absorption of sera.—Sera diluted with saline (1/8, vol/vol) were absorbed with concentrated CW at room temperature for 18 hours with rotary mixing; CW were removed by centrifugation.

Assay for DHR.—DHR to *C. parvum* and CW was assayed as described in (*19*).

Electron microscopy.—The CW preparations were routinely checked by negative staining with phosphotungstic acid and examined in a Philips 800 electron microscope.

^{125}I labeling of C. parvum *and CW.*—Tyramine was coupled to *C. parvum* and CW by the technique described in (*20*). *C. parvum* and CW were then dialyzed against saline and water, respectively. The tyramine incorporated into CW was estimated by the Folin assay (*13*). The protein contents of CW before and after tyramine coupling were 3.1 and 18%, respectively. Of the tyramine-coupled *C. parvum* and CW suspensions (7 mg/ml), 5 ml was washed 2–3 times in saline and finally resuspended in 0.4 ml phosphate-buffered saline; 2 mCi of ^{125}I (Radiochemical Centre, Amersham, Buckinghamshire, England) was added, and addition of 0.4 ml chloramine T (5 mg/ml) then followed immediately. After 5 minutes, 0.4 ml sodium bisulfite (25 mg/ml) and, immediately thereafter, 0.4 ml sodium iodide (10 mg/ml) were added. The suspension was washed once by centrifugation in saline and resuspended to its original volume. It was dialyzed for 5 days against daily changes of 2 liters of saline at 4° C, by which time the radioactivity in the dialysate had stabilized. In different batches only 0.01–0.02% iodine remained unbound, and no appreciable amount was released after incubation of the labeled *C. parvum* for 24 hours at 37° C. A total of 0.1 ml 10% thiomersalate preservative/10 ml suspension was added. Suspensions were stored at 4° C.

Gamma counting.—Organs taken from mice given injections of ^{125}I-labeled *C. parvum* were placed directly into plastic tubes, and the radioactivity was measured with a Packard automatic γ-counter.

Mice.—Male or female CBA-T6T6 mice, 8–12 weeks of age, were used.

Tumors.—The origins and techniques for maintenance and harvest of syngeneic M4 fibrosarcoma and RI leukemia were described in (*21*) and (*22*), respectively.

Measurement of tumors.—Appropriate dilutions of M4 cells were injected sc into a hind footpad by means of a 27-gauge needle. Subsequent tumor growth in the footpad was measured as footpad thickness in 0.1-mm units with a Schnelltester dial gauge caliper AO2T (H. C. Kroplin, Schlüchtern, Hasse, Federal Republic of Germany).

Irradiation of tumor cells.—M4 cells were irradiated (10,000 rads) with the use of a ^{137}Cs source.

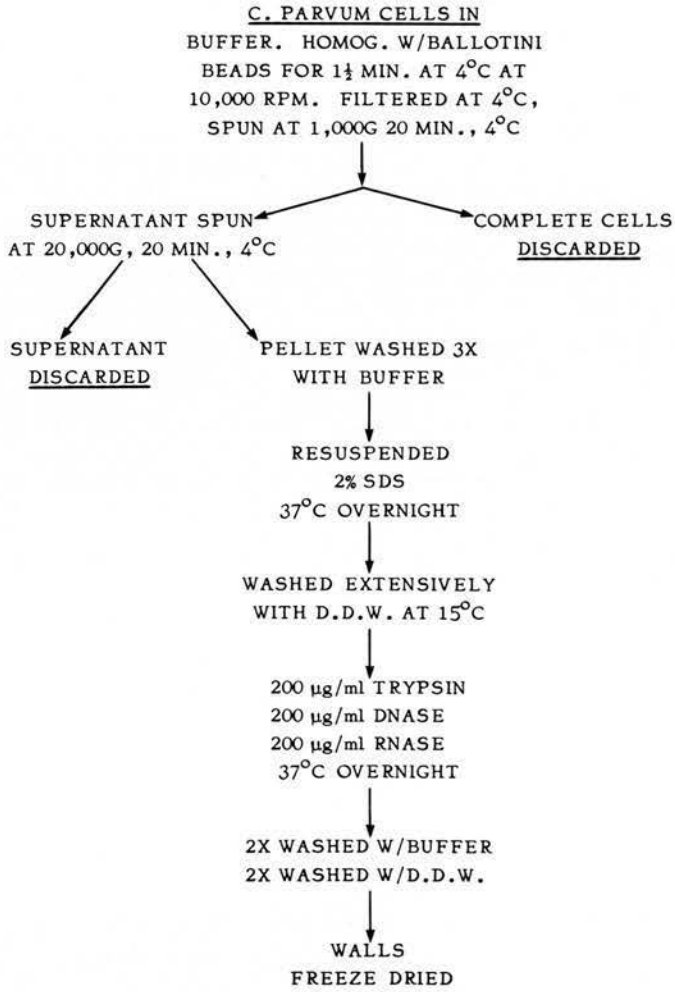
Cytostatic macrophage assay.—Peritoneal macrophages were prepared and assayed for their ability to inhibit the DNA synthesis of RI leukemia cells in vitro as described in (*22*).

Statistics.—The results were expressed as means (±SE). Means were compared by Student's *t*-test, and values of $P<0.05$ were considered significant.

RESULTS

Composition of CW, Peptidoglycan, and Carbohydrate

The CW, prepared as summarized in text-figure 1, were routinely checked for contamination by complete cells. CW did not take up gram stain, whereas complete



TEXT-FIGURE 1.—Scheme of preparation of *C. parvum* CW. Homog.=homogenized; W/=with; D.D.W.=double-distilled water.

TABLE 1.—*Chemical composition of CW and fractions*

CW and fractions	Protein %	Total sugar %	Hexosamine %	Total amino acids %	Phosphorus %
Complete CW	2.5	14.5	29.3	24.8	0.25
Peptidoglycan	0.0	7.5	17.8	31.4	0.0
Polysaccharide	0.0	20.0	24.3	0.8	0.0

cells were positive. CW stained with phosphotungstic acid and examined under the electron microscope appeared transparent and maintained the cell shape with only a few holes. Preparations that contained either complete cells or shattered CW were discarded. The chemical analysis of the complete CW is shown in table 1. The CW contained virtually no phosphate and little protein. Hexose, galactose, glucose, and mannose were identified by paper chromatography as components of CW. Uronic and aminohexuronic acids were also detected, but they were not quantified; these may be expected to influence the overall composition of the CW presented in table 1.

The two major components of the CW (peptidoglycan and carbohydrate) can be separated by treatment with dilute acetic acid, which produces a soluble polysaccharide (mol wt, ≈30,000 daltons) and a peptidoglycan shell that retains the cell shape and is resistant, like *C. parvum* and CW, to lysozyme (data not included). Table 1 shows the composition of the peptidoglycan and polysaccharide fractions. The polysaccharide had negligible amounts of amino acids. The peptidoglycan fraction still contained some sugars. Since peptidoglycans do not usually contain sugar (*23*), possibly some sugars from the polysaccharide were still bound to it. Repeated acetic acid hydrolysis removed the sugars but also hydrolyzed part of the peptidoglycan, inasmuch as diamino pimelic and alanine were also released.

TABLE 2.—*Amino acids and amino sugars in CW and fractions*

Amino acids and amino sugars	Complete CW		Peptidoglycan		Polysaccharide
	μmoles/mg	Molar ratio	μmoles/mg	Molar ratio	μmoles/mg
Glutamic acid	0.48	1	0.69	1	0.004
Muramic acid	0.57	1.18	0.80	1.16	0.004
Glycine	0.43	0.90	0.52	0.75	0.008
Alanine	0.62	1.29	0.76	1.10	0.008
Diamino pimelic acid	0.47	0.98	0.55	0.80	0.005
Glucosamine	0.19	0.40	0.31	0.45	0.028
Galactosamine	Modified		Modified		0.019

The amino acid and amino sugar compositions of CW, peptidoglycan, and polysaccharide are shown in table 2. The galactosamine had a slightly different retention time from that of the standard galactosamine used to calibrate the amino acid analyzer. It may therefore be modified, but this has not been investigated further.

Assays for Lymphoreticular Stimulatory and Antitumor Activities

Splenomegaly.—Mice were given ip injections of 400 μg CW or various doses of *C. parvum*. Spleens were weighed 14 days later at about the peak of the response to *C. parvum*. (*24*). Normal spleens weighed 0.087±0.005 g. *C. parvum* markedly increased spleen weight (0.48±0.03 g), but CW were not active (0.091±0.004 g).

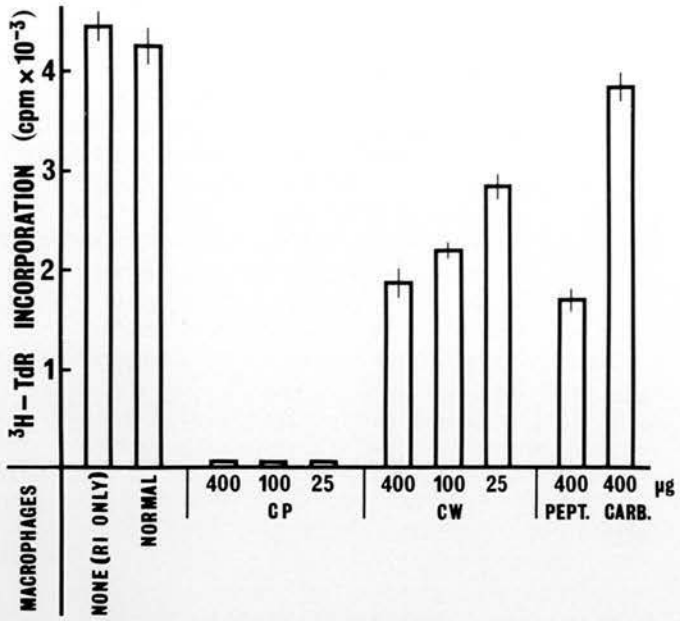
Growth of mixture of M4 cells with C. parvum *or CW.*—Mice were inoculated sc in the footpad with 10^6 M4 cells alone or mixed with 10 or 100 μg of *C. parvum* or CW (table 3). *C. parvum* suppressed tumor outgrowth at all tested concentrations. (The significant footpad swelling at day 20 following injection of M4 and 100 μg *C. parvum* was attributable to the *C. parvum;* it subsequently subsided.) CW was significantly suppressive at only the 100-μg dose and even then prevented tumor outgrowth in only 2/6 animals.

Intratumor injection of C. parvum *or CW.*—Mice were inoculated with 10^5 M4 cells in the footpad and 1 day later received 10 or 100 μg of *C. parvum* or CW in the inoculated footpad (table 3). CW failed to inhibit tumor

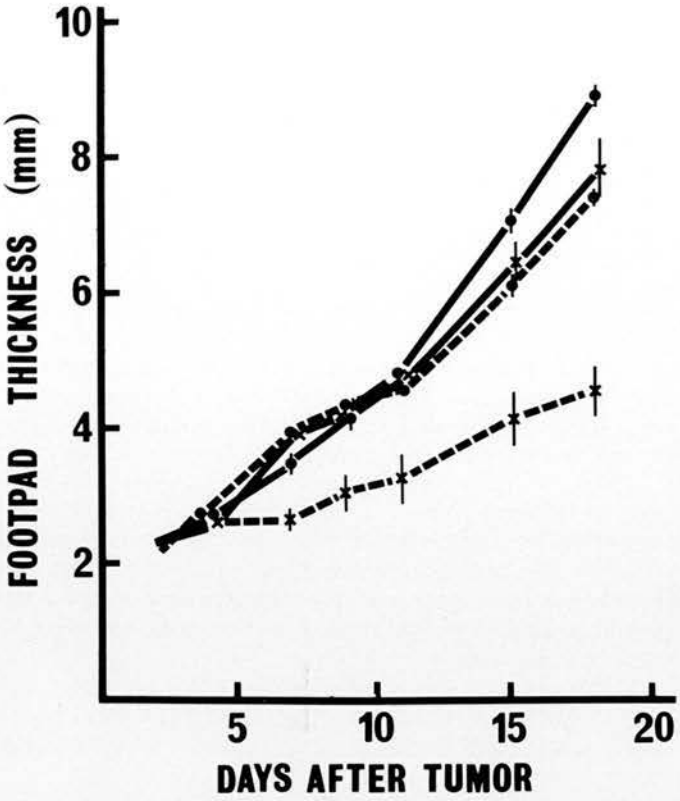
TABLE 3.—*In vivo antitumor activity of* C. parvum *and CW*[a]

Bacterial material	Dosage μg	Tumor outgrowth from *C. parvum*-CW plus tumor cell mixtures, mm mean±SE[b]	Intratumor injection of *C. parvum*-CW, mm mean±SE[c]	Immunization with *C. parvum*-CW irradiated tumor cell mixtures, mm mean±SE[d]
C. parvum	100	3.5±0.01 (0/6)	2.3±0.1 (0/5)	2.1±0.1 (1/5)
	10	2.7±0.07 (0/6)	2.6±0.08 (0/5)	1.9±0.05 (0/5)
CW	100	2.1±0.02 (4/6)	6.9±0.6 (5/5)	4.9±0.2 (5/5)
	10	4.0±0.3 (6/6)	6.6±1.1 (5/5)	5.5±0.4 (5/5)
None		4.5±0.1 (6/6)	6.9±0.7 (5/5)	4.9±0.4 (5/5)

[a] Measurements were taken 20 days after M4 injections. *Numbers in parentheses:* No. mice with tumors/No. in experimental groups.
[b] Mice were inoculated sc in the footpad with 10^6 live M4 cells alone (control) or mixed with 10 or 100 μg *C. parvum* or CW.
[c] Mice received sc injections in the footpad with 10^5 live M4 cells and 1 day later received 10 or 100 μg *C. parvum* or CW in the inoculated footpad. Control mice received no treatment.
[d] Mice were inoculated sc in the footpad with 5×10^5 irradiated M4 cells admixed with 10 or 100 μg *C. parvum* or CW; 7 days later, 10^5 live M4 cells were injected sc into the contralateral footpad.



TEXT-FIGURE 2.—Incorporation of tritiated thymidine (^{3}H-TdR) by RI leukemia cells growing alone or on monolayers of normal macrophages, or by macrophages from mice given injections of various doses of *C. parvum* (CP), CW, peptidoglycan (PEPT.), or carbohydrate (CARB.); cpm=counts per minute.



TEXT-FIGURE 3.—Growth of 10^6 M4 cells injected sc into a hind footpad of either 12 normal mice (●——●) or 12 mice sensitized 4 days earlier by sc injection of *C. parvum* into the contralateral foot (●--●). After 2 days of tumor growth, 6 normal (×—×) and 6 sensitized (×--×) mice were given injections of 70 µg CW directly into the tumor site.

growth, whereas both doses of *C. parvum* were fully effective.

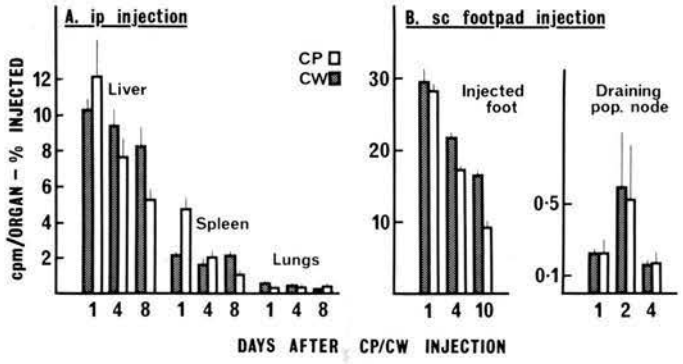
Immunization with irradiated M4 cells.—To test whether CW can stimulate specific cell-mediated immunity to tumor-specific transplantation antigen, mice were immunized with 5×10^5 irradiated M4 cells admixed with CW or *C. parvum* and challenged 7 days later in the contralateral footpad with 10^5 living M4 cells. Neither M4 cells nor *C. parvum* alone protected against tumor challenge, but, as expected (*25, 21*), the addition of *C. parvum* prevented tumor take (table 3). On the contrary, the addition of 10 or 100 µg of CW failed to suppress tumor outgrowth or inhibit tumor growth rate.

Cytostatic macrophages.—Mice were inoculated ip with different doses of *C. parvum* or CW or 400 mg of peptidoglycan or carbohydrate. Peritoneal macrophages were tested for cytostasis of RI leukemia cells 5 days later (text-fig. 2). Macrophages activated by *C. parvum* totally suppressed DNA synthesis of RI cells, but macrophages from mice given injections of CW were only partially inhibitory, in a dose-dependent manner. Of the two separated CW components, only the peptidoglycan was active.

Retention of C. parvum *surface antigen by CW.*—The CW preparations were shown to retain *C. parvum* surface antigens for both humoral and cellular immunity. The activity of hyperimmune mouse anti-*C. parvum* serum (agglutination titers, 1/2,000) was completely removed by adsorption with CW. CW were also capable of eliciting DHR in *C. parvum*-sensitized mice: Six days after a sensitizing sc injection of 70 µg *C. parvum* into a hind footpad, 70 µg of CW injected into the contralateral footpad elicited a 24-hour DHR footpad swelling of 0.3 ± 0.03 mm but was without effect in unsensitized controls.

Antitumor activity of CW in mice sensitized to C. parvum.—A component of the antitumor activity of intratumor *C. parvum* is mediated through a local immune DHR to *C. parvum* itself (*26*). In light of this and the ability of CW to elicit DHR reactivity to *C. parvum*, CW were injected intratumorally in mice previously sensitized and showing DHR to *C. parvum*. Mice were sensitized by sc injection of 70 µg *C. parvum* into a hind footpad. Four days later, 10^6 live M4 cells were injected into the contralateral footpad; intratumor CW were given 2 days later (text-fig. 3). Sensitization alone did not affect tumor growth, and intratumor CW were ineffective in nonsensitized mice. Tumor growth was, however, significantly inhibited by intratumor CW in sensitized mice. No complete regression occurred, and this contrasts with the 100% complete regression routinely achieved by equivalent amounts of intratumor *C. parvum* in the system (*see* table 3).

In vivo distribution and persistence of ^{125}*I-labeled* C. parvum *and CW.*—The in vivo fates of ^{125}I-labeled *C. parvum* and ^{125}I-labeled CW were compared to see whether the relative inactivity of the CW could be attributed to an altered distribution or persistence pattern. After ip injection of 300 µg of either ^{125}I-labeled



TEXT-FIGURE 4.—Distribution of ^{125}I-labeled *C. parvum* (CP) or CW injected either ip or sc into a hind footpad; cpm=counts per minute; pop.=popliteal.

TABLE 4.—*Effect of intratumor injection of CW attached to oil droplets*

Intratumor injection [a]	Tumor growth, mm mean±SE [b]
Untreated control	6.2±0.4 (5/5)
Oil alone	5.7±0.4 (5/5)
C. parvum (10 μg)	2.7±0.1 (0/5)
CW (10 μg)	5.2±0.2 (5/5)
Oil+CW (10 μg)	5.0±0.1 (5/5)
Oil+CW (70 μg)	4.7±0.4 (5/5)

[a] Mice were inoculated with 10^5 live M4 cells sc into the footpad, and 1 day later test substances (0.05 ml) were injected into the same site.

[b] Measurements taken 20 days after M4 injections. *Numbers in parentheses:* No. of mice with tumors/No. in experimental groups.

C. parvum or ^{125}I-labeled CW no overall difference in the uptake or persistence was observed in the liver, spleen, or lung (text-fig. 4A). Similarly, following sc injection into a hind footpad, the radioactivity profiles of both *C. parvum* and CW at the injection site and in the draining popliteal lymph node were not different (text-fig. 4B).

Intratumor injection of CW associated with oil droplets or emulsified in Freund's incomplete adjuvant.—M4 cells (10^5) were injected sc into the footpad; 1 day later CW attached to oil droplets, or appropriate control mixtures, were injected into the same site (table 4). The performance of CW–oil droplets was not significantly different from that of CW alone. Similar experiments with CW emulsified in Freund's incomplete adjuvant were also negative.

DISCUSSION

The characteristic lymphoreticular stimulatory and antitumor properties of *C. parvum* were not present or were drastically reduced in CW preparations. Our data on CW composition may be compared with those on the composition of other strains of "active" anaerobic coryneform bacteria analyzed previously (*27–29*). The qualitative composition of CW is similar, and close resemblances exist in amounts of CW amino acids and amino sugars.

The antitumor effects of *C. parvum* can conveniently be classified into the nonspecific, in the sense of not requiring an immune response to tumor antigens, and the specific, or adjuvant effect on tumor antigens (*3*). The residual antitumor effects of CW were nonspecific, being confined to macrophage activation and suppression of tumor outgrowth after injection of mixtures of M4 cells and CW. This latter effect is independent of a host immune response in that it is demonstrable in nude mice (*30*), in contrast to the antitumor effect of intratumor *C. parvum*, which is not expressed in T-cell depleted mice (*31, 32*). The low macrophage-activating capacity of CW preparations that we observed was attributable to the peptidoglycan moiety and could be sufficient to account for the variable antitumor effects of similarly prepared *C. parvum* CW reported in (*9*). Our results also agree with the report that *C. parvum* CW preparations failed to stimulate cell-mediated immunity to tumor antigens (*28*).

The in vivo distribution and persistence of *C. parvum* and CW were not different; manipulations designed to vary these parameters, i.e., emulsification in Freund's incomplete adjuvant and association with oil droplets, did not result in restored CW activity.

The almost total loss of activity of CW did not correlate with any detectable loss of antigenicity or immunogenicity. The role that immunity to *C. parvum* plays in the stimulatory and antitumor effects of *C. parvum* is equivocal. Specific *C. parvum* preimmunization has been shown to facilitate, but not be essential for, in vivo macrophage activation by *C. parvum* (*33*). Similarly, preimmunization has enhanced (*26*), or been without effect (*19*) on, in vivo antitumor effects in different experimental systems. Our present finding that CW retain *C. parvum* antigens but lack stimulatory and antitumor activity indicates that the stimulation resulting from immunity to *C. parvum* represented a minor contribution to the overall detected effects of *C. parvum*. That intratumor CW were detectably tumor-suppressive in mice presensitived to *C. parvum* is analogous to the reported local antitumor effects at the site of ongoing DHR reactions to other antigens that similarly lack any intrinsic stimulatory activity (*34*).

Clearly, CW are not solely responsible for the antitumor effect of *C. parvum*. Some limited activity has been attributed to a *C. parvum* carbohydrate fraction isolated from culture medium (*35*), and *C. parvum* phospholipid preparations are chemotactic for macrophages (*36*). The only fractions of *C. parvum* that have so far been shown to retain full antitumor activity are phenol-treated unbroken cells, which contain CW as well as an undefined amount of non-CW components (*10, 11*). It thus seems that complete antitumor activity is not associated with a single molecular component capable of being isolated in pure form: Synergism between, or molecular association of, CW and non-CW components may be required.

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Brief Communication: In Vivo Cortisone Sensitivity of Nonspecific Antitumor Activity of *Corynebacterium parvum*-Activated Mouse Peritoneal Macrophages [1,2]

Martin T. Scott [3,4]

SUMMARY—The nonspecific antitumor activity of *Corynebacterium parvum*-activated mouse peritoneal macrophages in vitro was inhibited by their prior treatment with cortisone acetate in vivo. Inhibition was marked (60%) 24 hours after cortisone injection and was still significant at 7 days, but antitumor activity was completely recovered by 9 days. This transient inhibition was sufficient to abolish the nonspecific protection afforded by *C. parvum* in an in vivo pretreatment model.—J Natl Cancer Inst 54: 789–792, 1975.

THE IN VIVO ANTITUMOR EFFECTS of *Corynebacterium parvum* (CP) are in some ways nonspecific and macrophage mediated (*1*). This paper describes the in vivo sensitivity to cortisone of the nonspecific antitumor activity of CP-activated macrophages. The antitumor activity of macrophages activated by CP is similar to that of those activated by protozoan infection (*2–5*), and my investigation was prompted by the recent description that the antitumor effects of macrophages activated by *Toxoplasma gondii* were abolished by in vitro treatment with hydrocortisone (*5*). Corticosteroid drugs figure in some cancer chemotherapy regimens, and a promising approach to the use of CP as an anticancer agent seems to be its use in either simultaneous or sequential combination with chemotherapeutic agents (*6–10*).

A previous study with the present mastocytoma tumor model demonstrated that the cell type among CP-stimulated peritoneal cells expressing nonspecific antitumor activity in vitro is the activated macrophage (*11*). This has been confirmed by two independent studies with other tumor models (*12, 13*).

MATERIALS AND METHODS

Mice.—Adult (C57BL/6×DBA/2)F_1 B6D2F_1 males were used throughout.

CP.—PX 374 was kindly provided by Burroughs-Wellcome and Co., Research Triangle Park, North Carolina.

Drugs.—Cortisone acetate (Sigma, St. Louis, Mo.), vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind.), and cyclophosphamide (Mead Johnson & Co., Evansville, Ind.) were used.

Tumor.—Mastocytoma P815 (MA), originally induced by 3-methylcholanthrene in a DBA/2 mouse, has been maintained in ascites form by weekly passage in B6D2F_1 mice for 2 years. Cells were harvested by peritoneal lavage with Hanks' balanced salt solution (HBSS) containing 10 U heparin/ml.

In vitro tumor inhibition assay.—Peritoneal cells were obtained by peritoneal lavage with HBSS containing 10 U heparin/ml. They were washed and resuspended in Eagle's minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (FCS) (Flow Laboratories Inc., Rockville, Md.). Target tumor cells were obtained as described above and resuspended in MEM 10% FCS. One ml peritoneal cells (10^6/ml) was added to 1 ml (10^5) MA cells in 16×125-mm tissue culture tubes (Falcon Plastics, Oxnard, Calif.). Cultures were incubated at 37° C in a 5% CO_2 atmosphere. After 24 hours of culture, 1 μCi tritiated thymidine (New England Nuclear Corp., Boston, Mass.) was added to each tube; 16 hours later, isotope incorporation into the acid-precipitable material was assayed as described in (*14*), except that Whatman glass fiber papers (2.5 cm GF/B) (H. Reeve Angel and Co. Ltd., London, England) were used.

RESULTS

Development of CP-Mediated In Vitro Tumor Inhibitory Activity

Groups of 5 mice were given 700 μg CP intraperitoneally (ip) 1, 2, or 4 days before removal of their peritoneal cells for assay of tumor inhibitory activity as described. Control cultures contained MA cells alone or MA plus peritoneal cells from normal mice (text-fig. 1). Normal peritoneal cells did not significantly modify the growth of MA cells in vitro. CP-stimulated peritoneal cells were significantly inhibitory 24 hours after CP injection (51% inhibition) and, after 4 days, inhibition of MA growth was almost total (97% inhibition). Cultures of either normal or CP-activated peritoneal cells alone were always less than 500 counts per minute (cpm) and not significantly different.

Cortisone Sensitivity of Antitumor Activity

Mice received 700 μg CP ip and, 4 days later, 2.5 mg cortisone acetate subcutaneously (sc) in the neck. At various times after cortisone treatment, peritoneal exudates were harvested and assayed for inhibition of tumor growth in vitro (text-fig. 2). The results are expressed as percent tumor inhibition calculated as follows:

$$\frac{\text{cpm/culture MA alone} - \text{cpm/culture MA + peritoneal cells}}{\text{cpm/culture MA alone}} \times 100.$$

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[2] This work was performed during the tenure of a Fellowship from the Cancer Research Institute, Inc., 1225 Park Ave., New York, N.Y., and was supported by Public Health Service contract NO1-CP23221 from the National Cancer Institute.

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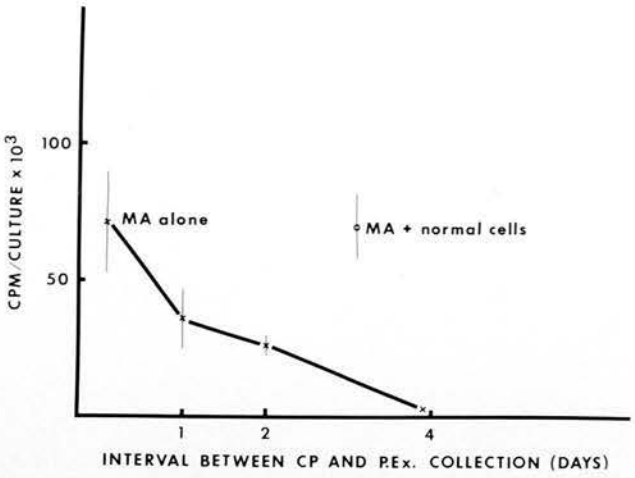
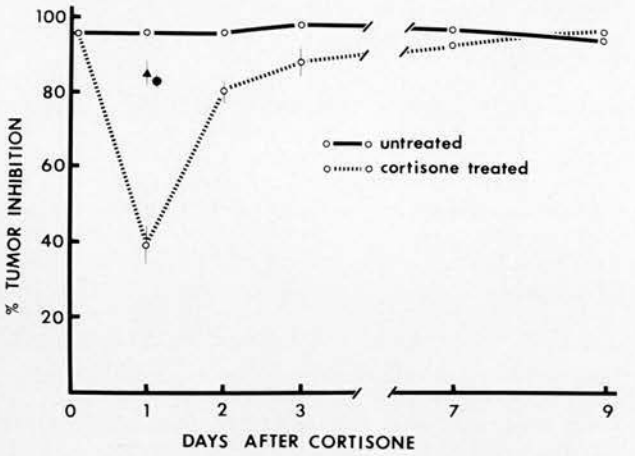
[4] The excellent technical assistance of Sandra L. Warner is gratefully acknowledged.



TEXT-FIGURE 1.—In vitro growth inhibition of MA cells by CP-activated peritoneal cells. Thymidine uptake, expressed as cpm/culture of 10^5 MA cells cultured with 10^6 peritoneal cells from normal mice (○) or mice that had received 700 μg CP ip 1, 2, or 4 days earlier (×). *Vertical bars* represent ± SE.



TEXT-FIGURE 2.—Cortisone sensitivity of in vitro antitumor activity, expressed as percent tumor inhibition (*see* text). 10^5 MA cells were cultured with 10^6 peritoneal cells harvested at various times from mice given either 700 μg CP ip (○——○), or 700 μg CP ip and 2.5 mg cortisone acetate sc in the neck 4 days later (○----○). Included are two groups of mice given 700 μg CP ip and, 4 days later, either 4 mg cyclophosphamide (▲) or 1 mg vinblastine sulfate (●) intravenously (iv). *Vertical bars* represent ± SE.

(Since the amount of isotope incorporated by the peritoneal cells relative to the tumor cells was negligible, total radioactive counts were used in calculating tumor inhibition.)

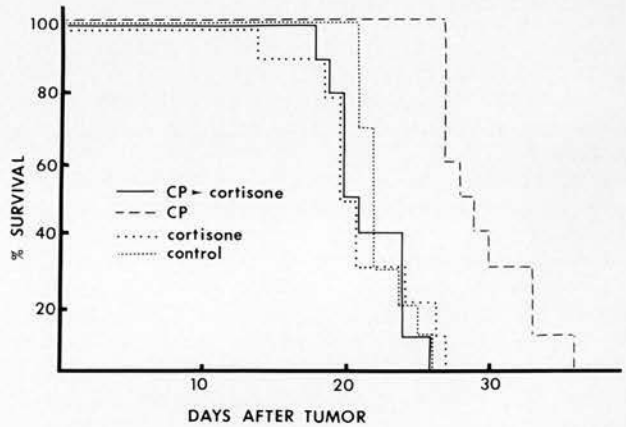
CP pretreatment alone produced the expected marked inhibition of tumor growth which persisted throughout the 9 days of the experiment, and cortisone treatment of these mice transiently reduced the antitumor effect. One day after cortisone injection it was reduced by 60%, still significantly reduced at 7 days (5%), but completely recovered by day 9. Cultures of peritoneal cells from non-CP-treated animals given injections of cortisone behaved as normal cells and did not modify MA growth. Included in text-figure 2 are the results from CP-treated mice that, instead of receiving cortisone, were given 1 mg vinblastine sulfate or 4 mg cyclophosphamide iv. Peritoneal cells were harvested 24 hours later. Inhibition of the CP antitumor effect by these two agents, though statistically significant, was considerably less than that of cortisone. All three drugs at their respective doses were equally able to reduce the number of cells during a 24-hour period, as measured by decrease in spleen weight: cortisone acetate, 50% reduction; vinblastine sulfate, 51%; cyclophosphamide, 43.8%.

Histologic Studies

Aliquots of peritoneal cell suspensions prepared as for culture were allowed to settle onto glass cover slips for 1 hour at 37° C. The overlying fluid was then pipetted off and the adherent monolayer fixed for 10 minutes in ice-cold methanol and stained with Giemsa. The number of peritoneal cells obtained from CP-treated or CP-cortisone-treated mice 24 hours after cortisone was given did not differ significantly, and the proportion of adherent cells in both these groups was greater than 90% macrophages. During the 1-hour incubation period, CP-activated macrophages spread out and were highly vacuolated, whereas normal macrophages remained rounded up. These changes are indicative of the activated state (*12*). CP-activated macrophages were apparently still activated 24 hours after cortisone treatment as judged by their rapid spreading and degree of vacuolation.

In Vivo Demonstration of Cortisone Sensitivity

To ascertain whether the magnitude of the transient inhibitory effect of cortisone was sufficient to be significant in vivo, the following experiment was performed. A group of 10 mice was given 700 μg CP ip; 4 days later they received 2.5 mg cortisone acetate sc in the neck, and the following day, 10^5 live MA cells ip. Control groups received only CP or cortisone or were untreated before challenge. Survival of the mice is shown in text-figure 3. Cortisone treatment did not



TEXT-FIGURE 3.—Survival of groups of mice challenged with 10^5 MA cells ip. Pretreatment for the groups was 700 μg CP ip 5 days before challenge (— — —) or, in addition, 2.5 mg cortisone acetate sc 1 day before challenge (———). Control groups received either cortisone acetate alone (. . . . . .) or were untreated (– – – –).

modify growth of the tumor in untreated mice; the mean survival times of control and treated groups were 22.8±0.7 (SE) and 21.2±1.2 days, respectively. CP significantly prolonged survival time (mean survival time, 29.7±1 day) but this effect was completely abolished by subsequent cortisone treatment (mean survival time, 21.6±0.8 days).

DISCUSSION

Macrophages activated by CP nonspecifically inhibit tumor cell growth in vitro (*11–13*). That such a nonspecific system is operative in vivo is suggested by the observation that the in vivo antitumor effects of CP are in large part resistant to various immunosuppressive procedures—T cell deprivation (*11*, *15*), antilymphocyte serum and splenectomy (*16*), and irradiation (*17*). The present data show that this nonspecific antitumor activity of the CP-activated macrophage is sensitive to in vivo treatment with cortisone. The effect was transitory but sufficient to abolish the protective effect of CP in an in vivo pretreatment model. Preliminary results suggest that the antitumor activity may not be equally sensitive to two other commonly used cytostatic agents—cyclophosphamide and vinblastine sulfate.

That the cortisone was administered when the nonspecific antitumor activity was fully developed, and no histologic differences were apparent between the peritoneal exudates of cortisone-treated or nontreated, CP-stimulated mice, indicates the cortisone acted at the effector cell level—the activated macrophage. The data do not, however, exclude the possibility that cortisone also may affect other cell types or interactions. The cytostatic activity of cortisone may be excluded, since the expression of tumor inhibition by CP-activated macrophages is highly radioresistant (*11*); further, both vinblastine sulfate and cyclophosphamide are also cytostatic. Insight into the mode of action of cortisone may be gained from recent work of Hibbs (*5*). Using macrophages activated by *T. gondii* infection, he demonstrated that their nonspecific tumor inhibitory activity was effected by direct contact between the activated macrophage and the target tumor cell. Lysosomes from the activated macrophage were secreted directly into the cytoplasm of the tumor cell, which subsequently lysed. Corticosteroid drugs prevented exocytosis of macrophage lysosomal enzymes, most probably through stabilization of the macrophage cell membrane (*18–20*), and treatment of *T. gondii*-activated macrophages in vitro with hydrocortisone inhibited their expression of antitumor activity (*5*). The present findings suggest that activated macrophages are similarly sensitive to cortisone treatment in vivo. Such a mechanism would also explain the relative lack of activity of cyclophosphamide and vinblastine sulfate, neither of which is a membrane-stabilizing drug.

The impetus for the present cortisone studies came from the predicted in vivo incompatibility between cortisone and CP-activated macrophages based on Hibbs' work; also, both clinical (*9*, *10*) and experimental (*6–8*) results with CP in combination with various chemotherapeutic agents suggest this to be a promising approach to its clinical use. Despite the transitory nature of the inhibitory effect of cortisone, it significantly impaired the performance of CP in vivo. Further studies to evaluate the potential cytostatic effect of corticosteroids as opposed to their possible inhibition of macrophage effector functions are recommended. It may prove possible, through manipulation of the corticosteroid therapy and nonspecific immunotherapy, to maximize the antitumor effect of each while minimizing the antagonistic effect of corticosteroids on macrophage activity. Since other cytostatic agents without membrane-stabilizing properties may prove less antagonistic, another consideration may be substitution of these drugs for corticosteroids if these are equally effective in the given tumor situation. The nonspecific antitumor effects of macrophages activated by BCG (*21*) are similar to those activated by CP (*11*, *12*) or protozoan infection (*2–5*), and the same drug sensitivity may be predicted.

In vitro models of the nonspecific antitumor activity of activated macrophages such as that described would seem to provide a ready screen for possible future drug incompatibilities. Such incompatibilities may be particularly important in advanced cancer patients, since these are most likely to be relatively anergic and depend on the nonspecific component of the antitumor activity of CP rather than any augmentation of specific tumor immunity; further, they are most likely to require continuous maintenance chemotherapy.

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CHEMO-IMMUNOTHERAPY OF MOUSE TUMOURS USING CYCLOPHOSPHAMIDE AND *C. PARVUM*

M. T. Scott

ABSTRACT

Treatment of a chemically induced mouse solid fibrosarcoma using either non-specific (*C. parvum* 350 μ i.v.), or specific active (s.c. *C. parvum* mixed with 5.10^5 irradiated tumour cells) immunotherapy, 4 days after a single dose of cyclophosphamide (200 mg/kg) was synergistically more effective than either *C. parvum* or drug treatment alone. A contributory factor may be that cyclophosphamide pretreatment has been shown to potentiate the specific antitumour immunity that arises from *C. parvum* interaction with tumour antigen.

Systemic *C. parvum* before cyclophosphamide will potentiate the antitumour effects of the drug — previously ineffective low doses becoming effective. No similar potentiation of the effects of another alkylating agent, Melphalan, was evident.

Despite the immunosuppressive nature of most cancer chemotherapy drugs, animal studies show that immunostimulation using *C. parvum* (CP) can be successfully combined with drug therapy, often with additive, or even synergistic effects (2-6). This report describes some preliminary experiments aimed at analysing the principles involved in such chemo-immunotherapy situations.

MATERIALS AND METHODS

10^6 live M4 tumour cells (1) were injected s.c. into a hind footpad of syngeneic CBA mice, and the resulting solid tumour was measured as footpad thickness using a dial gauge caliper. Footpads > 10.0 mm became necrotic and measurements were discontinued. CP (Wellcome) was diluted in saline, and cyclophosphamide (CY-Koch Light Labs. Ltd.) was dissolved in distilled water and 4 mg (200 mg/kg) injected i.p.

RESULTS

Experiment 1

Tumours were established and 6 days later CY was injected i.p. and CP (350 μg) was given i.v. 1, 4, 8 or 12 days after CY (Table I). CP at these times in non CY-treated mice was ineffective. The inhibitory effects of CY alone were transient, all tumours being > 10.0 mm and necrotic by day 20. Only CP 1 or 4 days after CY was effective and, of these, CP 4 days after CY was significantly better.

Experiment 2

After 4 days, tumours were CY treated, and 4 days later CP (70 μg) was injected directly into the tumour lesion (Table II). Neither CY, nor CP alone,

Table I. Antitumour effects of i.v. CP at various times after CY.
*$p < 0.05$ between results (Student t test)

Treatment	Tumour size at day 20 (footpad thickness mm ± s.e.)
Tumour alone day 0	>10.0
4 mg i.p. CY + 6	>10.0
350 μg CP i.v. + 7/10/14/18	>10.0
CY + 6, CP i.v. +7	7.8 ± 0.38*
" , " " + 10	6.4 ± 0.40*
" , " + 14	9.5 ± 0.37
" , " + 18	>10.0

Table II. Antitumour effects of intralesional (i.l.) CP after CY treatment

Treatment	Proportion of mice tumour free
Tumour alone day 0	0/6
4 mg CY i.p. + 4	0/6
70 μg i.l. CP + 8	0/6
CY + 4, CP + 8	5/6

caused tumour regressions, but 5/6 tumours regressed completely when CP followed CY.

Experiment 3

To study the effect of CY in the antitumour activity of CP it was necessary to eliminate the direct antitumour effects of CY itself. This was achieved by injecting CY 4 days before tumour cells since the *in vivo* half-life of CY is considerably shorter. CP (350 μg) was given i.v. 1 day after tumour cells (Table III). CY pretreatment was without effect. CP 1 day after tumour cells inhibited tumour growth but was significantly more effective in CY pretreated animals.

Table III. Antitumour effects of i.v. CP in CY pretreated mice.
*$p < 0.005$ between results (Student t test)

Treatment	Tumour size at day 20 (footpad thickness mm ± s.e.)
Tumour alone day 0	8.8 ± 0.41
4 mg CY i.p. -4	8.3 ± 0.56
350 μg CP i.v. +1	5.8 ± 0.23*
CY -4, CP + 1	4.2 ± 0.34*

Experiment 4

CP (350 μg) was injected i.v. 2 days after tumour cells and 4 days later a tumour inhibitory dose (4 mg), or an ineffective dose (1 mg) of CY was given (Table IV). The individual antitumour effects of CP and 4 mg CY were additive. The 1 mg dose of CY, ineffective in normal mice, was significantly inhibitory after CP treatment.

Table IV. Effect of i.v. CP pretreatment on the antitumour effects of CY. *p < 0.005 between results (Student t test)

Treatment	Tumour size at day 20 (footpad thickness mm ± s.e.)
Tumour alone day 0	>10.0
350 µg CP i.v. + 2	8.9 ± 0.37
4 mg CY i.p. + 6	6.8 ± 0.42*
1 mg CY i.p. + 6	>10.0
CP i.v. + 2, 4 mg CY + 6	4.9 ± 0.35
CP i.v. + 2, 1 mg CY + 6	6.9 ± 0.37

DISCUSSION

The findings from the preliminary experiments accord with previous studies (2-6) in showing that stimulatory CP immunotherapy in combination with immunosuppressive drug therapy can produce additive, and even synergistic antitumour effects, in mice. They also affirm the idea that the timing between the two agents is a critical factor, and this parameter is under further investigation. Thus far it would seem that the basis of successful CP-CY combinations may include both a potentiation of CP effects by CY, and of CY effects by CP.

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General discussion

Woodruff: It's very important in the design of clinical trials to have some advance information about the sort of order in which to employ your various therapies, and the sort of thing that Dr Scott reports I think is very important for that reason. But I do think that one needs to look at quite a lot of tumours before you can extrapolate too confidently. I think I'm right in saying that when Currie and Bagshawe first described the synergistic effect of *C. parvum* and cyclo-

phosphamide they found an interval of 12 days between administration of agents was optimal. With a CBA/methylcholantrene induced sarcoma we found about 8 or 9 days was optimal. These figures vary somewhat from the 4 days Dr Scott found was optimal.

Scott: I think our 4 day interval here agrees with the Fisher data. The Currie and Bagshawe data was using intradermal *C. parvum* and so was probably very different. This does check out with another mouse system—we used the CBA radiation induced leukaemia system, and found the same answer. We are well aware that 4 days in a mouse is really not very meaningful when you are talking about patients. We therefore correlate it with measurable parameters, and although we talk about 4 days in a mouse as presented here, we have been following the effects of cyclophosphamide on such things as reduction of spleen weight and peripheral leucocyte count, and so far the 4 days in our system correlates with just immediately prior to the escape from the suppressive effects of cyclophosphamide as measured by peripheral leucocyte count... whereas 8 days would correlate with the over-shoot.

Salomon: I would like to ask if you have considered the possibility of looking at the dilution of *C. parvum* susceptibility in tumours which are made resistant by cyclophosphamide.

Scott: No, we haven't. What we're looking at now is resistant to adriamycin and vinblastine and so we are having a look at the effect of cyclophosphamide to see whether you can get any sensitising effect in that respect. We don't have a tumour at the moment which is insensitive to cyclophosphamide.

There's just one other point which I didn't make, but in the light of Dr Foster's data this morning concerning increased toxicity of *C. parvum*, we find that intravenous *C. parvum* treatment will increase the susceptibility to toxic effects of cyclophosphamide. We also find that cyclophosphamide pretreatment will increase the susceptibility of mice to the toxic effects of very high doses of intravenous *C. parvum*.

Cancer Immunol. Immunother. 6, 107–112 (1979)

Cancer Immunology and Immunotherapy

Analysis of the Principles Underlying Chemo-Immunotherapy of Mouse Tumours

I. Treatment with Cyclophosphamide Followed by *Corynebacterium parvum*

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Summary. *A CBA mouse fibrosarcoma (M4) growing as a solid tumour was treated with a single high dose (200 mg/kg) of cyclophosphamide (CY) followed by C. parvum (CP) immunotherapy. The optimal time for the administration of both nonspecific (CP IV) and active specific (SC CP-irradiated tumour cell mixtures) immunotherapy after CY was 4 days, compared with 1, 8, or 12 days. Optimal combinations of CY and CP were 'synergistic' in that they provided a stronger antitumour effect than the sum of the individual effects of the two agents. In keeping with the finding that a 4-day interval between CY and CP was better than only 1 day, studies in normal mice show that when CP is given early during the suppressive phase of CY the onset of both specific and nonspecific antitumour effects are delayed. CY increased the susceptibility of mice to the toxic effects of a subsequent high systemic dose of CP.*

Introduction

Animal studies indicate that the combination of chemotherapy with bacterial immunostimulants such as BCG or CP may be a promising form of cancer treatment, the individual antitumour effects being additive or even synergistic[1] (Fisher et al., 1976; Hattori and Yamagata, 1977; Pearson et al., 1975; Purnell et al., 1977; Sansing et al., 1977; Tagliabue et al., 1977). These studies have shown that the time interval between treatment with the chemotherapeutic agent and with the immunostimulant is important. This paper is concerned with the analysis of the principles underlying chemo-immunotherapy with cyclophosphamide (CY) given before CP. The reverse combination, CY after CP, is the subject of the accompanying paper (Scott, 1979). CY was chosen because its marked immunosuppressive properties were likely to accentuate any antagonism between immunostimulation and drug therapy, and because previous workers have shown synergistic effects when CP was combined with CY in mouse tumour models (Currie and Bagshawe, 1970; Fisher et al., 1975; Pearson et al., 1975; Purnell et al., 1977). Two forms of CP therapy have been used: CP injected systemically (IV) and specific active immunotherapy by SC injection of CP mixed with irradiated tumour cells. The mode of action of systemic CP is considered to be predominantly nonspecific (i.e., unrelated to tumour antigens) and mediated by CP-activated macrophages, whereas local interaction of CP with tumour antigen generates strong, specific T cell-mediated antitumour immunity (reviewed in Milas and Scott, 1978; Scott, 1978).

Materials and Methods

Mice. Adult CBA T6T6 were used throughout.

Cyclophosphamide Monohydrate (CY). CY (Koch Light Laboratories, Colnbrook, Buckinghamshire, England) was dissolved in sterile distilled water for injection. Unless otherwise stated, injections were of 200 mg/kg.

C. parvum. A formalin-killed suspension, strain CN6134 (Wellcome Research Laboratories, Beckenham, Kent, England), was diluted with sterile saline for injection.

Tumour. The origins and techniques for maintenance and harvesting of syngeneic M4 fibrosarcoma and RI leukaemia cells have been described elsewhere (Bomford, 1975; Olivotto and Bomford, 1974).

Tumour Measurement. A 27 g needle was used to inoculate groups of 6–8 mice SC in a hind footpad with live M4 cells. Subsequent tumour growth was measured 2–3 times per week as footpad thick-

1 The terms synergistic/synergism have been used in this paper to describe (a) situation(s) where the antitumour effects of a combination of two treatments are apparently greater than the sum of each treatment alone. The complexities of a true definition of synergism have been critically reviewed by Berenbaum, Clin. Exp. Immunol. **28**, 1 (1977)

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ness in 0.1-mm units, with the aid of a Schnelltaster dial gauge caliper AO2T (H. C. Kroplin, Schlüchtern, Hessen, West Germany).

Irradiation of Tumour Cells. M4 cells were irradiated (10.000 rads) with a ^{137}Cs source.

In vivo Neutralisation Assay (Winn). Popliteal lymph node cells were gently homogenised in Hank's Balanced Salt Solution by means of a ground glass homogeniser. Viable (trypan blue exclusion) cells were mixed in a ratio of 10 : 1 with viable M4 cells and the mixture injected SC into the flanks of mice that had received 400 rads whole-body irradiation 24 h earlier. These mice were then monitored for tumour growth.

In vitro Cytostatic Assay. The technique was as described by Scott (1974). Peritoneal cells were mixed in vitro in a ratio of 5 : 1 with RI leukaemic cells. After 24 h of culture ^{3}H-thymidine was added and its uptake by RI cells was assessed 24 h later. Limitations to this form of cytostatic assay are apparent from the work of Stadecker et al. (1977).

Statistics. Means and standard errors were calculated for all experimental groups and differences where $P < 0.05$ (Student's t-test) were considered significant.

Results

Optimal Timing for CY and Specific Active CP Therapy

We injected 10^5 live M4 cells into a hind footpad and 4 days later when the tumours were macroscopically visible, CY, 200 mg/kg (the previously determined highest, nontoxic dose maximally inhibiting tumour growth), was injected IP. One, 4, 8, or 12 days later a mixture of 3.5 μg CP and 5 × 10 irradiated M4 cells (previously determined doses and ratio for optimal therapy) was injected into the contralateral footpad (Fig. 1). CY alone produced a marked but transient inhibition of tumour growth and CP-irradiated M4 mixtures 1, 8, or 12 days after CY were without further significant effect. Mixtures injected 4 days after CY, however, were significantly more inhibitory than CY alone and in the experiment shown resulted in one complete tumour regression in the six mice. Mixtures given at the same times in non-CY-treated mice were without effect (data not included); however, when given earlier, within 0–2 days after tumour cells, they were inhibitory (see Fig. 3b).

Optimal Timing for CY and IV CP Therapy

We injected 10^6 live M4 cells into a hind footpad and 4 days later CY, 200 mg/kg, was injected IP. One, 4, 8, or 12 days later CP (350 μg – previously determined optimal therapeutic nontoxic dose) was injected IV (Fig. 2). CY alone caused a transient inhibition of tumour growth 4–9 days after injection, after which a growth rate similar to that in controls was resumed. IV CP given 8 or 12 days after CY (i.e., at the time of, or after, the escape of the tumour from the inhibitory effect of CY) was without effect. Tumours treated with IV CP 1 day after CY grew more slowly than those treated with CY alone, although this effect was not always statistically significant in repeated experiments. IV CP 4 days after CY was consistently more effective than either CY alone or

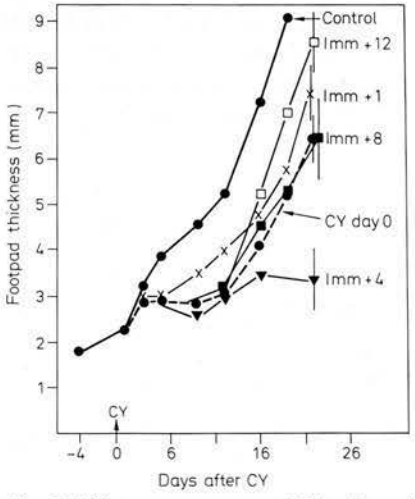

Fig. 1. M4 tumours were established in mice, which then received CY, 200 mg/kg IP (CY) 4 days later (●– – –●) or were untreated (controls) (●——●). Individual groups of CY-treated mice were then given CP-irradiated M4 mixtures SC (Imm) 1 (×——×), 4 (▼——▼), 8 (■——■), or 12 (□——□) days later

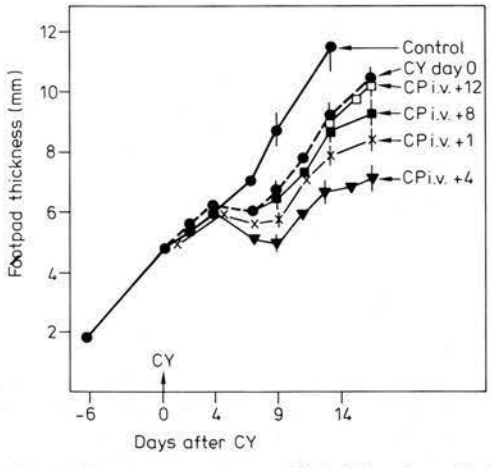

Fig. 2. M4 tumours were established in mice, which then received CY, 200 mg/kg IP (CY) 4 days later (●– – –●) or were untreated (controls) (●——●). Individual groups of CY-treated mice were then given CP IV 1 (×——×), 4 (▼——▼), 8 (■——■), or 12 (□——□) days later

CP 1 day after CY. CP given IV at the same times in non-CY-treated mice was without effect (data not included), but as with CP-irradiated tumour cell mixtures, they were effective against smaller tumour masses (see Fig. 3a).

Potentiation of CP-Mediated Antitumour Effects by CY Pretreatment

It seemed possible that CY pretreatment was modifying the host in such a way that the antitumour effects of CP given 4 days later were potentiated. To determine this it was necessary to eliminate the effects of CY on the tumour itself, and this was achieved by giving CY 4 days before injection of tumour cells, since the in vivo half-life of CY is only a few hours (Bach, 1975). We injected 10^6 live M4 cells into a hind footpad of normal mice or mice that had received CY 4 days earlier. One day later these mice were injected with 350 μg CP IV, or were untreated (Fig. 3a). CY pretreatment alone was without effect or, in some experiments, enhanced tumour growth (e.g., Fig. 3b). Growth in non-CY-treated mice was inhibited by CP, but the effect was significantly greater after CY pretreatment. The same protocol was used in further experiments where SC CP-irradiated tumour cell mixtures were substituted for IV CP (Fig. 3b). Again, therapy was considerably more effective in CY-pretreated animals.

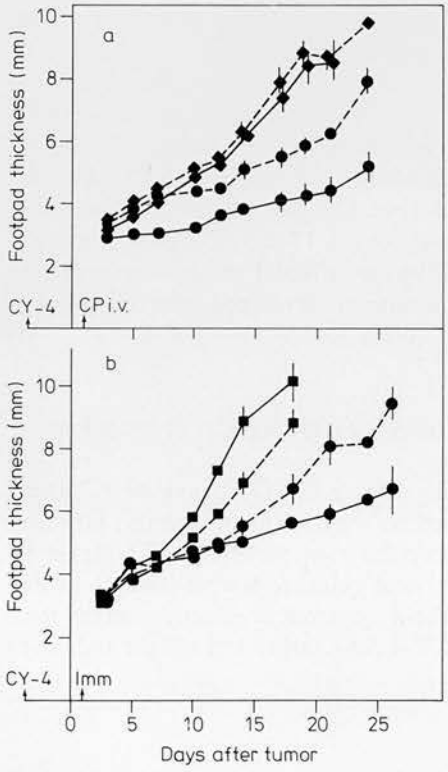

Fig. 3. a M4 tumors were established in either normal mice (controls) (♦– – –♦) or mice treated with CY, 200 mg/kg IP 4 days earlier. One day after tumour establishment mice from both groups received 350 μg CP IV or no further treatment. ●——●, CY and CP IV; ♦——♦, CY only; ●– – –●, IV CP only
b In the same scheme as **a**, SC immunization with 3.5 μg CP mixed with 5×10^5 irradiated M4 cells (Imm) was substituted for IV CP. ■——■, CY only; ■– – –■, controls; ●– – –●, Imm only; ●——●, CY and Imm

Kinetics of the Suppressive Effects of CY in Normal Mice

The finding that the antitumour effects of CP given 1 day after CY were less good than those of CP given after 4 days (see Figs. 1 and 2) suggested that the stimulatory effects of CP given shortly after CY may be suppressed. Figure 4 shows the suppressive effects of a single IP injection of CY in normal mice, as judged by spleen weight and peripheral blood leucocyte count. The effects on both parameters were roughly parallel: within 24 h both were significantly depressed, recovering after 4–5 days. By day 8 leucocyte counts were again normal, whereas spleen weights were above normal, returning to normal between 12 and 18 days after CY.

Effects of CY Pretreatment on CP-Mediated Specific and Nonspecific Antitumour Effects

The nonspecific assay was based on the accumulated reports that, after systemic CP, peritoneal cells acquire the capacity for, significant inhibition of the growth of various tumour cells in vitro, the phenomenon being attributable exclusively to CP-activated macrophages (Olivotto and Bomford, 1974; Scott, 1974). In the experiment shown in Table 1, mice were injected IP with CY and 1 or 4 days later received 350 μg CP IP. Fur-

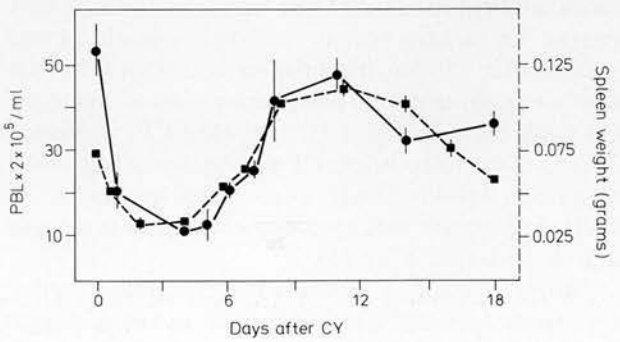

Fig. 4. Kinetics of the modification of peripheral blood leucocyte (PBL) count and spleen weight by a single IP injection of 200 mg CY/kg

Table 1. In vitro, nonspecific tumour inhibitory activity of peritoneal cells from mice treated with systemic CP after CY

Effector cells: Peritoneal cells from mice treated at days			Inhibition assay at days	
−4	−1	0	+4	+11
–	–	–	118,156 ± 6,302[a]	244,620 ± 48,309[a]
–	CY[b]	–	118,532 ± 420	180,724 ± 30,039
–	–	CP[c]	224 ± 48[d]	1,049 ± 198
–	CY	CP	3,762 ± 420[d]	756 ± 121
CY	–	CP	198 ± 60	–

[a] Results expressed as ^{3}H-thymidine incorporation cpm/culture. Incorporation by peritoneal cells alone was insignificant
[b] CY, 200 mg/kg IP
[c] CP, 350 μg IP
[d] Result significantly different from similarly designated results ($P < 0.0005$)

Table 2. Specific cell-mediated immunity arising from SC injection of CP-irradiated tumour cell mixtures after cyclophosphamide

Effector cells: Popliteal lymph node cells from mice tested at days			Winn assay at days	
−4	−1	0	+6	+12
–	–	–	8/8[a]	8/8[a]
–	–	Imm[b]	0/8	0/8
–	Cy[c]	Imm	8/8	0/8
CY	–	Imm	0/8	0/8

[a] Results expressed as proportion of recipient mice developing tumours
[b] Imm, SC 3.5 μg CP mixed with 5×10^5 irradiated M4 cells
[c] CY, 200 mg/kg IP

ther groups received either CY or CP alone, or were untreated. Peritoneal cells from the various groups were assayed for in vitro tumour inhibitory capacity 4 and 11 days after CP. No inhibition resulted from CY treatment alone. In non-CY-treated mice marked inhibition was apparent both 4 and 11 days after CP, whereas if CY was given 1 day before CP activity was significantly impaired at day 4 but had fully recovered by day 11. No similar impairment was apparent when CP was delayed until 4 days after CY.

Strong, specific, T cell-mediated antitumour immunity results from SC injections of CP mixed with irradiated M4 (Bomford, 1975) and other tumour cells (Scott, 1975), and is detectable in a Winn neutralisation assay when cells from the node draining the site of mixture injection are used as effector cells (Scott, 1975). In the experiment shown in Table 2, CP-irradiated M4 mixtures were injected SC into a hind footpad of normal mice or of mice treated with CY either 1 or 4 days earlier. Six and 12 days after immunisation, draining popliteal lymph node cells from the various groups were assayed for tumour inhibitory capacity by the Winn assay. Cells from normal immunized mice were fully inhibitory at both 6 and 12 days. CY 1 day before immunisation inhibited the 6- but not the 12-day effects, whereas CY 4 days before CP had no effect. Further control groups showed that no immunity developed after injection of either CP or CY alone.

Increased CP Toxicity Following CY Pretreatment

Mice injected with 350 μg CP IV 4 days after 200 mg CY/mg IP showed no overt signs of toxicity; however, an increased susceptibility to the effects of a higher IV CP dose (1.4 mg) was evident. Normal mice tolerated this CP dose without apparent ill effects, whereas mice pretreated with CY 4 days earlier lost weight and seven of ten died.

Discussion

The finding that CP can be combined with CY in the treatment of a mouse fibrosarcoma to give more pronounced effects than are obtained with either agent alone accords with the findings of other workers using various multiple- or single-dose regimens in different mouse tumour models (Currie and Bagshawe, 1970; Fi-

sher et al., 1975; Pearson et al., 1975; Purnell et al., 1977). To facilitate analysis of the interaction between CP and CY I have restricted the present study to treatment with a single injection of CY followed by a single injection of CP.

In the present model, the optimal timing of CP immunotherapy, both systemic and specific active, was 4 days after CY, compared with 1, 8, or 12 days. With specific CP therapy the 4-day interval was critical to obtain potentiated antitumour effects, but with systemic CP, although a 4-day interval was optimal, potentiated effects were also apparent with a 1-day interval. CP 8 and 12 days after CY, i.e., at a time when the tumour was escaping, or had escaped, from the inhibitory influence of the drug, was ineffective. Similar data have been reported for a mouse leukaemia where the optimal timing for systemic CP after CY was during remission, and CP given immediately before relapse was ineffective (Pearson et al., 1975).

When CP was injected 1 day after CY the expression of its stimulatory effects was delayed compared with CP given 4 days after CY. The relative sensitivities of macrophage activation and generation of specific immunity to the suppressive effects of CY, partial and total respectively, correlated with the partial and total loss of potentiated antitumour effects in vivo following either systemic or specific active CP therapy 1 day after CY compared with 4 days. CP is relatively resistant to degradation in vivo (Scott and Milas, 1977), and it may be presumed that the stimulatory effects of CP 'recovered' once the suppressive effects of CY had worn off because of persisting CP. Intact fluorescein-labelled CP is still present in the spleen and draining lymph node 4—5 days after IV and SC injection respectively (Scott and Milas, 1977).

A basis for the reduced effectiveness of CP given very shortly after CY is then postulated to be a delay in onset of the stimulatory effects of CP due to the persisting suppressive effects of CY. It is interesting that two recent studies where multiple weekly injections of CY and systemic CP have been used to treat two different mouse solid mammary adenosarcomata (Fisher et al., 1976; Purnell et al., 1977), the optimal CY-CP intervals were defined as 3 and 4 days, respectively, both intervals producing significantly better results than when CY and CP were given on the same day. At variance with these data are those of Currie and Bagshawe (1970), who in the treatment of a mouse solid fibrosarcoma, found a CY-CP interval of 12 days to be considerably better than 3, 6, 10, or 16 days. The CP was given ID, however, and this may be a determining factor.

A further factors contributing to the optimal CY-CP interval of 4 days with the M4 fibrosarcoma model is likely to be the demonstrated CY-mediated potentiation of the effect of both subsequent systemic and specific active CP therapy. The fact that the optimal timing for CP after CY coincided with the period immediately before recovery from the suppressive effects of CY suggests a possible basis for the potentiated CP effects, namely, that the cells contributing to the recovery after CY may, through modified metabolism, for example, be hyper-responsive to CP.

Overt toxicity was apparent only when very high doses of systemic CP were given after CY. However, the stimulatory effects of repeated low doses of CP are known to be cumulative (Scott and Warner, 1976), and the possibility of toxic effects should be a consideration in any repetitive dose regimens.

CY was chosen for the present study because its potent immunosuppressive effects were predicted to accentuate any antagonism between immunostimulant and immunosuppressive therapy. The findings seem to affirm what might have been previously presumed, i.e., that the best time for immunotherapy is likely to be after the drug toxicity for cells has worn off and before the tumour growth has gone out of control. Additive and synergistic antitumour effects in mice have been reported for a range of cancer therapy drugs when given in combination with CP (Fisher et al., 1976; Hattori and Yamagata, 1977; Pearson et al., 1975; Purnell et al., 1977; Sansing et al., 1977; Tagliabue et al., 1977). Since drugs used in cancer treatment differ in their immunosuppressive activities it will be interesting to see whether the same principles described here for CY apply to other drugs, particularly those of low or negligible immunosuppressive activity.

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Cancer Immunology and Immunotherapy

Analysis of the Principles Underlying Chemo-Immunotherapy of Mouse Tumours

II. Treatment with *Corynebacterium parvum* Followed by Cyclophosphamide

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Summary. *The nonspecific macrophage-mediated antitumour effects resulting from IV C. parvum (CP) in mice were resistant to cyclophosphamide (CY), whereas the specific T cell-mediated immunity resulting from SC injection of CP-irradiated tumour cell mixtures was sensitive for up to 8 days after immunisation, and resistant thereafter. Mice with solid fibrosarcoma (M4) were treated by IV CP followed by CY and, with intervals between CP and CY of 1–4 and 15–18 days, the antitumour effects of the CY were potentiated in a 'synergistic' manner. This did not happen with 8- or 12-day intervals. The most potent antitumour effects (39% complete regressions) occurred with the 15–18 day interval. (The antitumour activity of another alkylating agent, melphalan, was similarly potentiated when given 12–18 days after IV CP, but not after 4 days.) No potentiated antitumour effects were found when specific active immunotherapy with CP-irradiated tumour cell mixtures preceded CY by 1, 4, 8, 12, or 18 days. CP given IV increased the susceptibility of mice to subsequent high doses of CY, the peak sensitivity being around 10 days after CP.*

Introduction

In the preceding paper the optimal conditions for giving *Corynebacterium parvum* (CP) immunotherapy after CY were defined for the treatment of an experimental mouse solid fibrosarcoma (Scott, 1979). This paper considers the situation where the order of the two agents is reversed, i.e., CY after CP in the treatment of the same tumour.

Materials and Methods

The mice, tumours, assay system, CP, and CY were as described in the previous paper (Scott, 1979). Unless otherwise specified the CY injections were 200 mg/kg. Melphalan (Wellcome Research Laboratories, Beckenham, Kent, UK) was dissolved in the acid diluent provided.

Results

CY Resistance of CP-Activated Macrophages

The in vivo mediator of the immunologically nonspecific antitumour effects of CP is considered to be the CP-activated macrophage (reviewed in Milas and Scott, 1978; Scott, 1978). The nonspecific inhibition of tumour cell proliferation in vitro by CP-activated peritoneal cells is attributable exclusively to such activated macrophages (Olivotto and Bomford, 1974; Scott, 1974), and the CY sensitivity of this effector mechanism was examined (Table 1). At day 0 mice were injected with 350 μg IP, followed by CY IV at day +4. (By this time the nonspecific antitumour activity of CP-activated macrophages is fully established (Olivotto and Bomford, 1974; Scott, 1974). Four days later peritoneal cells were removed and assayed for their ability to inhibit the incorporation of ^{3}H-thymidine into syngeneic RI leukaemic

Table 1. Failure of CY to inhibit the nonspecific tumour-inhibitory activity of peritoneal cells stimulated by a previous injection of *C. parvum*

Effector cells: Peritoneal cells from mice treated at days		Inhibition assay
0	+4	day +8
–	–	113,467 ± 2,977[a, d]
–	CY[b]	112,983 ± 1,082[d]
CP[c]	–	4,085 ± 842[e]
CP	CY	2,587 ± 342[e]

[a] Results expressed as (^{3}H) thymidine incorporation cpm/culture. Incorporation by peritoneal cells alone was insignificant
[b] CY, 200 mg/kg IP
[c] CP, *C. parvum* 350 μg IV
[d, e] Results not significantly different from similarly designated results

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cells in vitro. CP-activated cells were markedly inhibitory, whereas inhibition after CY alone was slight and inconsistent. The inhibitory activity of CP-activated cells was undiminished following CY treatment.

CY Sensitivity of CP-Mediated Specific Antitumour Immunity

As described in the previous paper (Scott, 1979), the SC injection of CP mixed with irradiated tumour cells generates specific cell mediated antitumour immunity, which is detectable in a Winn assay performed with cells from the lymph node draining the injection site. To determine the CY sensitivity of this immunity, mice were immunised SC in both hind footpads with 3.5 μg CP mixed with 5×10^5 irradiated M4 tumour cells. Different groups of these mice then received CY IP 1, 4, 8, 15, or 22 days later, and the activities of the various popliteal lymph node cell populations were determined in a Winn assay 4 days after CY. For each time point the appropriate nonimmunised and immunised non-CY-treated controls were included; the results are set out in Table 2. CY 1 and 4 days after immunisation completely abolished immunity, but by day 8 immunity was becoming CY-resistant and it was completely so at days 15 and 22.

Recovery of CP-Mediated Specific Antitumour Immunity from the Inhibitory Effects of CY

Although, as described above (Table 2), CY given 4 days after immunisation abolished immunity in the draining lymph node 4 days later, this was demonstrated to have recovered fully 12 days later (Table 3).

Optimal Timing for CY after IV CP Therapy

Mice were injected with 10^6 live M4 cells SC into a hind footpad and 2 days later received 350 μg CP IV or were untreated. Groups of six mice, both CP-treated and control, received CY IP or no treatment 1, 4, 8, 12, or 18 days later. The size of the M4 tumour growing in the footpad was monitored throughout (Fig. 1a–d). CP IV at day +2 (tumour established day 0) caused a characteristic temporary inhibition of tumour growth (Scott, 1979). Inhibition of tumour growth by CY at day +6 in non-CP-treated mice was similar to the effects of CP at day +2, and the combination of the two was apparently additive (Fig. 1a). (The results with CY 1 day after CP were similar; the data have not been included.) CY at day +10 in non-CP-treated mice caused no detectable inhibition of tumour growth and when it was given in combination with CP no added effect was apparent (Fig. 1b). CY alone at day +14 was ineffective; however, a slight, but significant, effect occurred in CP-pretreated mice (Fig. 1c). CY alone at day +20 was ineffective, but when it was given in combination with CP at this point a marked inhibition of tumour growth occurred, with two complete regressions in the group of six mice (Fig. 1d). In repeat experiments the results were similar, the most potent antitumour effects and the only complete regressions (accumulated total 38.9 ± 5,6%) occurring when CY was given 15–18 days after IV CP.

Table 2. CY sensitivity of specific antitumour immunity resulting from SC injection of *C. parvum*-treated tumour cell mixtures

Treatment		Proportion of Winn assay mice tumour-free	
Imm[a] day 0	–	8/8	assay day +5
Imm day 0	CY[b] day +1	0/8	
–	–	0/8	
Imm day 0	–	8/8	assay day +8
Imm day 0	CY day +4	0/8	
–	–	0/8	
Imm day 0	–	7/8	assay day +12
Imm day 0	CY day +8	5/8	
–	–	0/8	
Imm day 0	–	6/8	assay day +19
Imm day 0	CY day +15	8/8	
–	–	0/8	
Imm day 0	–	5/8	assay day +26
Imm day 0	CY day +22	8/8	
–	–	0/8	

[a] Imm, SC injection of 3.5 μg *C. parvum* mixed with 5×10^5 irradiated M4 cells
[b] CY, 200 mg/kg IP

Table 3. Recovery of *C. parvum*-mediated specific antitumour immunity from the inhibitory effects of CY

Effector cells: Popliteal lymph node cells from mice treated at days		Winn assay at days:	
0	+4	+8	+16
Imm[b]	–	0/8[a]	0/8[a]
Imm	CY[c]	8/8	0/8
–	CY	8/8	8/8
–	–	8/8	8/8

[a] Results expressed as proportion of mice developing tumours
[b] Imm, SC injection of 3.5 μg *C. parvum* mixed with 5×10^5 irradiated M4 cells
[c] CY, 200 mg/kg IP

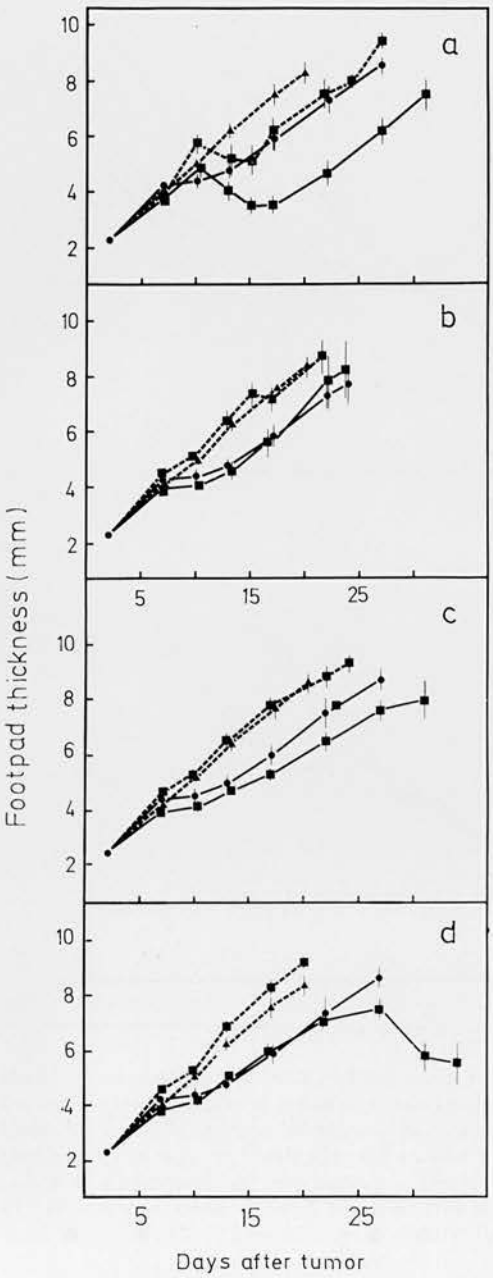

Fig. 1a–d. Optimal timing for CY after IV *C. parvum* therapy: M4 tumours were established in mice at day 0. At day +2 they received IV *C. parvum* (CP) or no treatment (controls). The two groups then received CY or no further treatment at days +6 (i.e., 4 days after CP) (**a**), +10 (i.e., 18 days after CP) (**b**), +14 (i.e., 12 days after CP) (**c**), or +20 (i.e., 18 days after CP) (**d**). ●—●, CP; ■—■, CP followed by CY; ■- - -■, CY; ▲- - -▲, controls

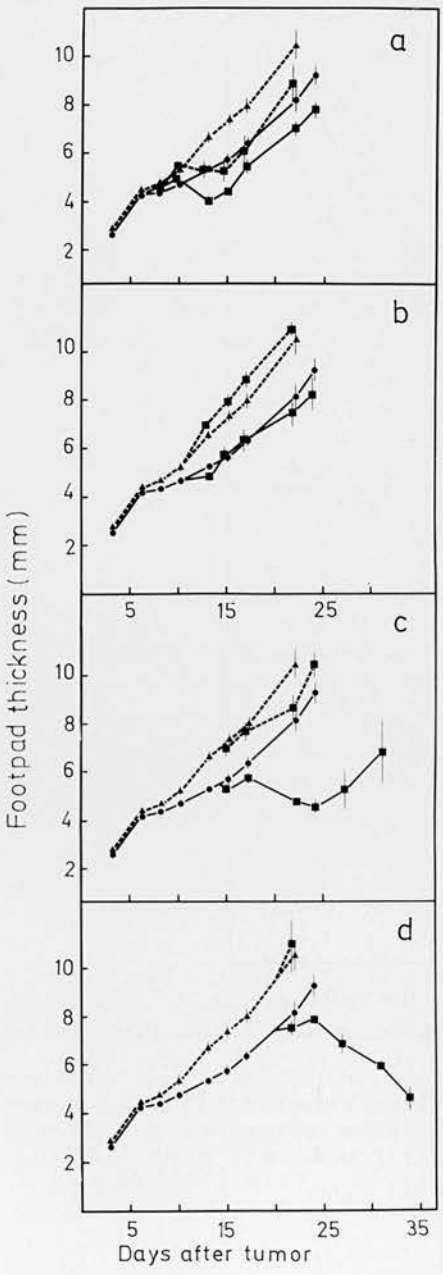

Fig. 2a–d. Optimal timing for melphalan after IV *C. parvum:* M4 tumours were established in mice at day 0. At day +2 they received IV CP or no treatment (controls). The two groups then received melphalan (Melph) or no further treatment at days +6 (i.e., 4 days after *C. parvum*) (**a**), +10 (i.e., 8 days after CP) (**b**), +14 (i.e., 12 days after CP) (**c**), or +20 (i.e., 18 days after CP) (**d**). ●—●, CP; ■—■, CP followed by Melph; ■- - -■, Melph; ▲- - -▲, controls

Optimal Timing for Melphalan after IV CP Therapy

For reasons discussed later, the experiments were repeated with another alkylating agent, melphalan, instead of CY. Melphalan was injected IP in a dose of 10 mg/kg 4, 8, 12, or 18 days after IV CP (Fig. 2a–d). Melphalan alone at day +6 (tumour day 0, CP IV day +2) caused a temporary inhibition of tumour growth similar to that caused by CY (cf. Fig. 1a). The combined effects of CP and melphalan were also additive (Fig. 2a). Melphalan alone at day +10 was ineffective, and no additive effects were seen when it was combined with CP (Fig. 2b). Mel-

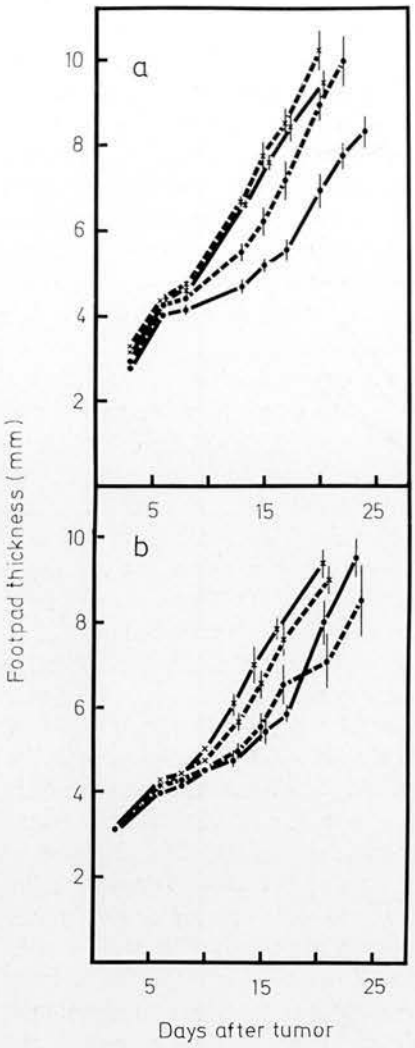

Fig. 3a and b. M4 tumours were established in mice at day 0. At day +2 they received IV CP or no treatment (control). At day +6 control and CP-treated mice were either not treated or given CY, 50 mg/kg IP (**a**) or Melph 5mg/kg IP (**b**). ●—● CP followed by CY (**a**) or Melph (**b**); ●---●, CP; ×—× CY (**a**) or Melph (**b**); × --- ×, controls

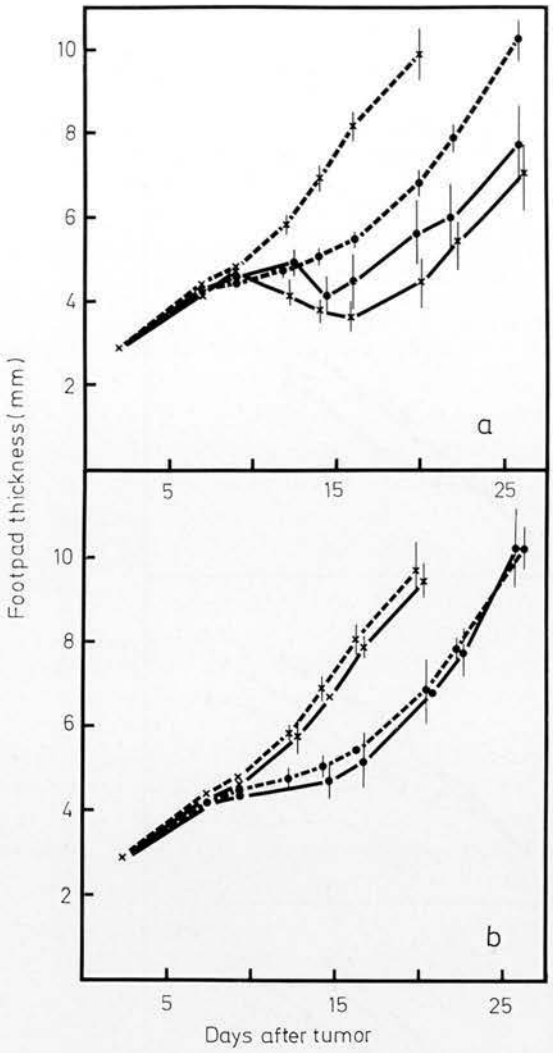

Fig. 4. Failure of active specific CP therapy to potentiate the effects of CY: M4 tumours were established in mice at day 0. At day +2 they were immunised by a single SC injection of 3.5 μg CP mixed with 5 × 10^5 irradiated M4 cells (Imm) or received no treatment (control). Control and immunised mice then received CY or no further treatment at days +6 (i.e., 4 days after Imm) (**a**) or +20 (i.e., 18 days after Imm) (**b**). ●—● Imm followed by CY; ●---●, Imm; ×—×, CY; × --- ×, controls

phalan alone at day +14 was ineffective, but in CP-treated mice a marked inhibition of tumour growth occurred. This inhibition was, however, temporary and all tumours regrew (Fig. 2c). Melphalan alone at day +20 was ineffective, but in CP-treated mice its strongest anti-tumour effects (decrease in tumour size) were recorded at this time (Fig. 2d). All tumours eventually regrew, but in a repeat experiment one of six regressed completely in this group, while none completely regressed in the other groups.

Potentiation of Subeffective CY and Melphalan Doses by IV CP Pretreatment

Low doses of CY (< 50 mg/kg) caused no measurable inhibition of tumour growth in the M4 model, but such doses were effective following IV CP. As seen in Fig. 3a, tumours were established by SC injection of 10^6 live M4 cells in the hind footpad, and 2 days later 350 μg CP was injected IV. Four days after CP, 50 mg CY/kg was injected IP. Control mice either received CP or CY

Table 4. Increased susceptibility to the toxic effects of CY on mice pre-treated with IV *C. parvum*

Cyclophosphamide day 0 IP	*C. parvum* pretreated (350 μg IV)					
	Day −21	−14	−10	−4	−1	None
300 mg/kg	1[a]	2	10	3	4	2
400 mg/kg	3	5	10	7	8	3

[a] Number of mice dead out of group of 10

alone, or were untreated. Similar results were obtained with 25 mg CY/kg. Fig. 3b shows the same experimental protocol but with a subeffective dose of melphalan (5 mg/kg i.e., only half the effective dose; see Fig. 2) instead of CY. No CP-mediated potentiation was observed.

Failure of Specific Immunization with CP-Irradiated Tumour Cell Mixtures to Potentiate the Effects of CY

Experiments identical in design with those shown in Fig. 1 except that specific active immunotherapy (SC injection of 3.5 μg CP mixed with 5×10^5 irradiated M4 cells) was substituted for IV CP were performed. No additive or synergistic[1] antitumour effects were obtained when this form of CP therapy preceded CY by 1, 4, 8, 12, or 18 days. The data for the 4- and 18-day time intervals are shown in Figs. 4a and b. With the 4-day interval the individual antitumour effects of immunisation and CY were not even additive.

Increased Susceptibility to the Toxic Effects of CY following IV CP Pretreatment

One, 4, 10, and 21 days after 350 μg CP IV, groups of mice were given either 300 or 400 mg/kg CY IP (Table 4). Increased sensitivity to CY was apparent between 1 and 10 days after CP, peaking at 10 days. Between 14 and 21 days it returned to normal.

Discussion

The predominant antitumour activity following systemic injection of CP is considered to be immunologically nonspecific and effected by CP-activated macrophages, whereas after local interaction of CP with tumour antigen (e.g., SC injection of CP-mediated tumour cell mixtures) tumour destruction is mediated by specific T lymphocytes (reviewed in Milas and Scott, 1978; Scott, 1978). The preceding paper (Scott, 1979) has described how both these forms of CP therapy may be given after CY in the treatment of a mouse solid fibrosarcoma to achieve additive and even synergistic antitumour effects. In the present study the reverse situation, where CP preceded CY in the treatment of the same tumour, has been analysed.

CY given 4 days after IV CP did not modify the nonspecific tumour inhibitory activity of peritoneal macrophages, whereas the specific cell-mediated immunity expressed in the draining lymph node following SC injection of CP-irradiated tumour cell mixtures was abolished. CP-mediated specific antitumour immunity was CY-sensitive until about 8 days after immunisation, after which it became CY-resistant. These kinetics most probably reflect the transition of the lymphocytes expressing specific tumour immunity from a proliferating to a nonproliferating population. Using a different mouse model, Tuttle and North (1976) similarly found that 7 days after local injection of CP mixed with live tumour cells, the specific T cell-mediated immunity expressed in the draining lymph node was sensitive to the antimitotic drug vinblastine sulphate. The abolition of tumour-specific immunity by CY given 4 days after immunisation was not permanent, and the immunity had spontaneously and fully recovered by 12 days after CY. This may be attributed to the recovery of a residual population of specific immune cells, or a restimulation resulting from persisting CP and tumour antigen, as discussed in the preceding paper (Scott, 1979).

Potentiated antitumour effects were obtained when the M4 solid fibrosarcoma was treated with IV CP followed by CY. The appearance of these effects was biphasic, occurring when CY was given 1 or 4 days and 15–18 days after CP. During the early period tumours were sufficiently small to be measurably affected by the usual high dose of CY, and the effects of CP and CY were apparently additive. However, the fact that low, subeffective doses of CY were effective after CP allows the CP-CY interaction to be defined operationally as synergistic. This also applies to the later period, when the larger tumours were apparently unaffected by high-dose CY. Here the effect was not just due to the smaller tumours in CP-treated mice being susceptible to CY, since tumours of similar size in non-CP-treated mice were too large to be measurably affected by CY (cf. Fig. 1c and 1d).

To investigate a possible basis for these CY-CP interactions, the drug melphalan was substituted for CY. There are data showing that CP pretreatment of mice

1 The terms synergistic/synergism have been used in this paper to describe (a) situation(s) where the antitumour effects of a combination of two treatments are apparently greater than the sum of each treatment alone. The complexities of a true definition of synergism have been critically reviewed by Berenbaum, Clin. Exp. Immunol. **28**, 1 (1977)

with systemic CP inhibits the microsomal enzyme activity of the liver (Macnee and Nimmo-Smith, 1978; Soyka et al., 1976). CY requires microsomal enzyme activation to effect its antitumour activity, and its metabolism has been shown to be modified by systemic CP pretreatment (Fisher et al., 1976b), suggesting a basis for the observed potentiation effects. Melphalan is an alkylating agent that has an inhibitory effect on M4 similar to that of CY but is intrinsically active, i.e., does not require liver activation. The biphasic nature of the combined antitumour effects when melphalan followed IV CP resembled those seen with CY. Unlike the situation with CY, however, during the early phase the combined effects of CP and melphalan were not synergistic, but only additive, since IV CP did not allow the use of subeffective doses of melphalan. This suggests that a CP-mediated modification of liver enzyme activation of CY may contribute to the observed potentiation of CY given shortly after IV CP. With regard to the observed difference between melphalan and CY, it is interesting that in the treatment of a mouse solid mammary carcinoma with repeated combinations of either CY or melphalan 3 days after systemic CP, Fisher et al. (1976a) reported significantly greater antitumour effects with CY.

In the present study, with the longer time interval between IV CP and drug treatment, the effects of both CY and melphalan were similarly potentiated, suggesting that a non-drug-specific mechanism was operating. An hypothesis under experimental consideration is that drug-mediated damage to the tumour, particularly the necrosis and ensuing inflammation, whilst themselves insufficient to produce an observable decrease in tumour size, may serve to focus antitumour-active macrophages concentrating their activity at the tumour site. Certainly the biphasic occurrence of the IV CP-mediated potentiation of the antitumour activity of CY is in keeping with different mechanisms operating during the different periods.

No potentiated antitumour effects occurred when specific active immunotherapy with SC injection of CP-mediated tumour cell mixtures preceded CY by 1, 4, 8, 12, or 18 days. The early stages of the development of specific tumour immunity have been shown to be inhibited by CY, and this would account for the fact that during this period the antitumour effects of immunisation and CY were not even additive. With a longer CP-CY interval, however, the generation of specific immunity was resistant to CY but there were still no potentiated antitumour effects resulting from combined treatment. Differences between the characteristics of the antitumour activity resulting from systemic and local CP therapy have been detailed elsewhere (Milas and Scott, 1978; Scott, 1978) and the in vivo interaction with CY may now be added to these.

The strongest antitumour activity, with an accumulated total of 38.9% complete regression, occurred when CY was given 15–18 days after IV CP. The previous study showed that when CY preceded CP the time interval for optimal therapy was only 4 days (Scott, 1979), and these differences should be a consideration in the design of clinical chemo-immunotherapy protocols where the two agents are likely to be administered asynchronously and repeatedly, i.e., involving both chemo-immunotherapy and immuno-chemotherapy intervals.

Clinical findings have suggested that CP may reduce drug toxicity (Israel and Edelstein, 1975), but here mortality resulting from very high doses of CY was increased by CP pretreatment. It may be significant that the optimal CP-CY interval for tumour therapy did not coincide with this period of increased CY sensitivity. Systemic CP has also been reported to increase the susceptibility of mice to a range of other cancer chemotherapy agents – 5-FU, melphalan, and methotrexate (Fisher et al., 1976a; Foster, 1978).

In addition to alkylating agents, additive or synergistic antitumour effects have been demonstrated when CP has been combined with nonalkylating antitumour drugs (Fisher et al., 1976a; Hattori and Yamagata, 1977; Tagliabue et al., 1977) and it will be interesting to see whether similar principles will apply to these combinations as have been described here for CP combined with CY.

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Corynebacterium parvum as an Immunotherapeutic Anticancer Agent

Martin T. Scott

IMMUNOLOGIC INTEREST in the gram-positive anaerobe *Corynebacterium parvum* was aroused following the demonstration of its remarkable stimulatory effect on the reticuloendothelial system when administered as a killed vaccine.[1] Subsequent work describing its antitumor properties in animals has led to the current clinical interest in the organism as a potential anticancer agent in humans. This article reviews the accumulating data on animal tumor therapy and also the preliminary clinical studies. An intelligent appraisal of the antitumor properties of *C. parvum,* however, first requires some review of its stimulatory effects reported in nontumor systems, and these are discussed first.

EXPERIMENTAL PROPERTIES

Macrophage Activation

Following i.v. injection of killed *C. parvum,* the organisms are taken up by the cells of the reticuloendothelial system, resulting in a prolonged hyperphagocytic response due to the emergence of large numbers of highly activated macrophages.[1] Manifestations of this stimulation are splenomegaly, hepatomegaly, increased lung weight, and augmented clearance of particulate matter from the bloodstream.[1-4] Macrophages activated by *C. parvum* respond in a manner similar to those activated by other means (see below) in that they show accelerated adherence to glass, vacuolation, and increased lysosomal enzyme activity.[6,7] The augmented phagocytic capability and lysosomal enzyme content contribute to their increased bactericidal capacity, which has been demonstrated in vitro,[8] and it is this nonspecific mechanism that is most probably responsible for the resistance against various bacterial and protozoal infections in vivo.[9,10]

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Humoral Immunity

C. parvum has been shown to be an adjuvant in the classical sense that it boosts antibody response to a variety of antigens.[11-18] Both IgM and IgG antibody levels are augmented,[18] and relative binding affinities are enhanced.[11,16] A change in the class of antibody following *C. parvum,* the production of IgE, has been reported;[19] but interestingly the only strain of *C. parvum* to do this was inactive as a reticuloendothelial stimulant. Many commonly used adjuvants, e.g., Freund's complete adjuvant and *Bordetella pertussis,* require thymus-processed lymphocytes (T cells) to effect their activity,[20-23] whereas *C. parvum* is capable of boosting antibody levels to T-cell-independent antigens.[15,17,18] Investigations into the cellular basis for the adjuvant effects of *C. parvum*[15,18] and other adjuvants[24] indicate that it is the activated macrophage that provides the added proliferative stimulus to the antigen-sensitive, and, ultimately, antibody-producing, B lymphocyte. A further role for macrophages activated by *C. parvum* and other adjuvants is considered to be that they cause chronic retention of lymphocytes within lymphoid organs, thereby facilitating extended contact between the antigen-charged phagocytes and lymphocytes, effectively magnifying the antigenic stimulus.[25,26]

Cellular Immunity

Systemic injection of *C. parvum* has been shown to depress various T-cell-mediated immune phenomena. Thus far, delayed hypersensitivity,[10,27-30] phytohemagglutinin (PHA), mixed lymphocyte, graft-vs.-host,[31] and homograft[32] reactivities have been reported impaired. An immunosuppressive effect on *Trichinella spiralis* infection has also been attributed to depression of T-cell immunity by *C. parvum.*[33] Since antitumor immunity is recognized as being predominantly cell-mediated, the finding of T-cell depression by an antitumor agent seems anomalous and deserves consideration. Insight into a possible underlying cellular mechanism and yet another role for the *C. parvum*-activated macrophage came from in vitro studies of the T-lymphocyte response to the mitogen PHA.[34] It was shown that spleen cells from *C. parvum*-stimu-

lated animals were refractory to stimulation with PHA; however, after removal of macrophages the T lymphocytes were again fully responsive. The conclusion was that T-cell activity was being inhibited by *C. parvum*-activated splenic macrophages. In contrast, cells from lymph node that had been directly stimulated with local injections of *C. parvum* were still capable of responding to optimal amounts of PHA,[34] indicating that T-cell inhibition does not occur in the lymph node. These findings have their parallel in some recent in vivo work describing depression of delayed-type hypersensitivity (DTH) by pretreatment with *C. parvum.*[30] DTH resulting from a subcutaneous injection of sheep red blood cells was only depressed by *C. parvum* pretreatment if the *C. parvum* was injected intravenously (i.e., stimulating the spleen); subcutaneous *C. parvum* (stimulating the lymph nodes) was without effect even when injected into the same region as the subsequent injection of sheep red blood cells. The apparently unique role of the *C. parvum*-stimulated spleen in mediating the depression phenomenon was confirmed by splenectomy experiments, the mechanism being interpreted as being attributable to an effective depletion of DTH-reactive T cells due to their sequestration and possible destruction in the *C. parvum*-stimulated spleen.[30] The apparent non-involvement of the lymph node in depression of cell-mediated immunity is important to the consideration of *C. parvum* as an antitumor agent, since strategic local injections of *C. parvum* will be described to be effective in tumor therapy. Another relevant finding was that systemic *C. parvum* needed to precede antigen sensitization to effect its DTH-depressive activity,[30] and since treatment of established tumors means that sensitization with tumor antigens must in large part precede *C. parvum,* some depression of cell-mediated immunity may thereby be circumvented.

While it is not the intent of this section to be comprehensive, some other effects reported for *C. parvum* are listed: protection against viral infection[35] and graft-vs.-host disease,[36] induction of autoimmunity evidenced by hemolytic anemia,[37-39] fetal resorption,[40] an increased sensitivity to endotoxin[43] and histamine.[44]

Species of Corynebacteria

The antitumor and other effects of several species of anaerobic corynebacteria other than *C. parvum* appear in the literature. Two things should be borne in mind: first, the taxonomy of the corynebacteria remains a matter of considerable controversy;[3,45-47] second, several species have been demonstrated to stimulate the reticuloendothelial system to greater or lesser degrees, while others are inactive.[3,4,48] The two strains figuring most prominently in current tumor work are *C. parvum* and *C. granulosum,* and these are apparantly similar in their reticuloendothelial-stimulating and antitumor effects. There are specific references comparing the antitumor activities of different strains.[12,35,48,49]

ANIMAL TUMOR THERAPY STUDIES

The purpose of Table 1 is to provide a source of references as to where *C. parvum* has been used, and under what circumstances in the various animal tumor models to date. Until recently the majority of animal investigators have restricted themselves to prophylaxis with *C. parvum,* i.e., stimulation of the animal prior to tumor challenge, or they have taken only a relatively cursory look at therapy situations. Prophylactic models have been invaluable in elucidating the possible modes of antitumor action of *C. parvum;* however, this review restricts consideration of the animal data primarily to therapy models using either syngeneic or semisyngeneic tumors.

Therapy Using Systemic C. parvum

The first demonstration that a systemic injection of *C. parvum* was effective against an established syngeneic tumor was in 1966 by Woodruff and Boak.[50] They demonstrated delayed appearance of a subcutaneously injected spontaneous mammary adenocarcinoma by a single i.v. injection of *C. parvum* 8 or 12 days after tumor injection. Once the tumor became palpable, however, the subsequent growth rate was unaffected.[50] Later studies by the same group showed that a single i.p. injection of *C. parvum* 3 days after tumor establishment inhibited the actual growth of the same adenocarcinoma and two unrelated methylcholanthrene-induced sarcomata,[51] and also that the vaccine was equally effective in T-cell-deprived mice.[48,52] Another spontaneous mammary carcinoma, growing as a solid tumor, was inhibited by biweekly i.p. injections commencing either at the time of tumor injection[53] or 14 days after tumor establishment.[54] Studies in this laboratory have shown a marked but transitory inhibition of growth of a solid mastocytoma (P-815) in the mouse foot-

Table 1. References to the Various Uses of *C. parvum* in Animal Models

Tumor Designation	Host*	Prophylaxis		Therapy†		Combination
		Systemic	Local	Systemic	Local	
Mammary carcinoma	A-strain (s)	50,49,69	69	50,69,51, 48, 52		Antitumor globulin (69,51)
Mammary carcinoma	C3H/He (s)	57‡				
Mammary carcinoma	DBA/2 (s)			53,54		
Sarcoma (methylcholanthrene)	CBA X A (s)	50				
Sarcoma (methylcholanthrene)	C57BL/6J (s)		59		59	
Sarcoma (methylcholanthrene)	CBA (s)			51,48	60	Cyclophosphamide (60, 48), antitumor globulin (51), stilboestrol (48)
Sarcoma (methylcholanthrene)	A/HeJ (s)			51,48		Mitomycin-C/neuraminidase-treated tumor cells (48)
Sarcoma (methylcholanthrene)	C57BL (s)		62		62	
Sarcoma (methylcholanthrene)	C3H (s)	57‡,67	57‡	57‡		Whole-body irradiation (67)
Sarcoma (meth A)	BALB/c (s)	73				
Sarcoma J	Allogeneic mice	84		84,35		
CBAT-3 Fibrosarcoma	CBA (s)	80				
ICIGCI$_1$ (dimethylbenzanthracene)	DBA/2 X C57BL/6 (ss)	12‡	12‡			
Leukemia AKR	AKR (s) or F_1 (ss)	58,74		58		
Leukemia L-1210	C57BL/6 X DBA/2 (ss)	12‡	12‡	56		
Leukemia Moloney	CD F_1				64‡	BCNU (64)
Leukemia YC8	BALB/c (s)			85		
Leukemia L-5178	BALB/c X DBA/2 (ss)	80				
Leukemia RI (radiation)	DBA (s)	80				Irradiated tumor cells (80)
Leukemia Eổ G2	DBA/2 X C57BL(s)	66,87		65,66,87		Procarbazine (65)
Leukemia (myeloblastic, chloroma)	Outbred rats			53	53	Formal killed tumor cells (53)
Hepatoma H-129	Allogeneic mice	80				
Hepatoma (ethionine)	Hooded rats (s)			68	68	Surgical excision & irradiated tumor cells (68)
Plasmacytoma PC6	BALB/c (s)	80				
Adenovirus-12	CBA (s)		86			Cell-free tumor extract (86)
Lewis lung tumor	DBA/2 X C57BL/6 (ss)	12‡	12‡			
Melanoma—Fortner's	Hamster, allogeneic				63	
Mastocytoma P-815	C57BL X DBA/2 (ss)	55		55	61	
Ehrlich ascites	Allogeneic mice	72‡,84		84,35		

*Refers to inbred mouse strains unless shown otherwise; s = syngeneic, ss = semisyngeneic.

†For the purposes of this table, treatment commenced on the same day as tumor challenge is included under therapy.

‡Strains of corynebacteria other than, or in addition to, *C. parvum* used.

pad when *C. parvum* is given i.v. 2 days after tumor establishment.[55]

With regard to nonsolid tumors, failure of multiple i.p. *C. parvum* injections to influence the growth of a mouse leukemia (L-1210) has been reported by Mathé.[56] An i.p. injection of *C. granulosum* 2 days after i.v. injection of fibrosarcoma cells into mice significantly reduced the number of metastases in the lungs and prolonged survival of the mice. *C. granulosum* 7 days after tumor injection did not affect lung metastases, although survival of the mice was still prolonged.[57] Lamensans et al. have shown that i.p. *C. parvum* 1 day after i.p. AKR leukemia cells had little effect on the ultimate survival of mice.[58]

The systemic effects against established solid sarcomas can be achieved either by i.v. or i.p. injection of *C. parvum,*[51] and the favored use of the i.p. route in animal studies is presumed to be because of its convenience. Doses of *C. parvum* employed for systemic injection into mice, whether single or multiple, have varied from 100-1,000 μg dry weight of organisms, the most frequent dose being around 500 μg per injection. Toxicity in mice has been reported for both i.v. and i.p. routes of injection for extremely high doses[48] and for more routinely used doses.[55,57,59,60] There is a paucity of published data on the relative efficiency of various doses of systemic *C. parvum* in therapeutic models; the author's work using a mouse mastocytoma showed the maximum tumor-inhibiting effect to be with 700 μg i.v., at which dose some deaths occurred due to toxicity. Lower doses were not toxic but were considerably less effective at inhibiting tumor growth. Multiple doses of *C. parvum* were no more effective than a single dose and considerably more toxic.[55] An increase in toxicity with multiple doses of *C. parvum* has been reported elsewhere,[59] as has the apparent lack of increased antitumor effect with multiple doses.[56] There is one report, however, that multiple doses were better than a single injection against a mammary carcinoma.[51]

Despite the undeniable tumor suppression described in experiments using systemic *C. parvum* therapy alone, the effects are transient, and no complete regressions have been recorded.* This is in contrast to the regressions that have occurred after some local injections of *C. parvum* and *C. parvum* in combination with other forms of therapy discussed below.

Therapy Using Local C. parvum

No toxicity problems have been encountered following the local administration of *C. parvum.*† Injections have been either subcutaneous or intradermal, neither of which results in marked splenomegaly or hepatomegaly, stimulation being restricted to the lymph nodes draining the sites of *C. parvum* injection. Because of this localized effect the relationship between the site of *C. parvum* injection and the tumor site becomes a relevant consideration.

There is a report that an intradermal injection of *C. parvum* into the flank opposite an existing solid fibrosarcoma caused significant depression of tumor growth.[60] Other investigators using a mouse mammary adenocarcinoma[54] and mastocytoma[61] found that local injection of *C. parvum* at sites distant from the tumor were only minimally effective, but when injected near to the tumor site (i.e., at a site likely to engage the tumor-draining node) marked inhibition of growth occurred. Interestingly, both studies suggest that local *C. parvum* injection near to the tumor site may be more effective than systemic injections. Significant growth inhibition of a solid fibrosarcoma following ipsilateral s.c. injections of *C. parvum* has also been reported.[59] Subcutaneous injection of *C. parvum* into the inguinal and axillary regions reduces simulated pulmonary metastases resulting from i.v. injection of fibrosarcoma cells, even if delayed up to 14 days.[62] In none of the cases cited have any complete tumor regressions been reported.

C. parvum has also been injected directly into the tumor lesion itself.[53,54,61,63] Preliminary experiments injecting mixtures of live mouse mammary carcinoma cells with *C. parvum* resulted in normal tumor growth for 14 days and then a rapid and complete regression.[53] Later studies employed a intralesional injection of *C. parvum*

*Some complete and lasting regressions of mouse solid tumors following systemic *C. parvum* or *C. granulosum* therapy have since been reported.[88]

†While doses of *C. parvum* under 4 mg are well tolerated subcutaneously in mice, even 1.4 mg intradermally may cause local granulomata in man or rodents (Dept. of Clinical Immunology, Wellcome Research Labs). The side effects from injection of 2-10 mg/sq m subcutaneously in man have been limited to local discomfort and low grade fever.[89]

14 days after tumor establishment, and this caused rapid and lasting regressions of both the primary tumor and its metastases.[54] Studies in this laboratory using a solid mastocytoma in mice have shown that an intratumor injection of *C. parvum* up to 12 days after tumor establishment causes some complete regressions.[61] Similarly, regression of primary tumors and inhibition of development of pulmonary metastases occurs following intralesional injection of *C. granulosum* into established hamster melanomas.[63] All mice cured by intralesional injection of *C. parvum* thus far have been shown to be highly and specifically immune to tumor rechallenge.[53,54,61]

Doses (200–500 μg) for local injection of *C. parvum* into mice, whether single or multiple, have been similar to those given systemically. Studies with the mouse mastocytoma have shown that an intralesional injection of 70 μg was optimal, higher and lower doses resulting in fewer complete regressions.[61] This reduced effectiveness of high doses of *C. parvum* may be attributable to overstimulation of the draining lymph node.

Combination Therapy

The combination of immunostimulation using corynebacteria with cytoreductive chemotherapeutic agents has produced some interesting and promising results. Currie and Bagshawe[60] followed i.v. cyclophosphamide cytoreductive therapy with intradermal *C. parvum* injection and produced dramatic inhibition of growth of established solid fibrosarcoma with 70% lasting regressions. Neither cyclophosphamide nor *C. parvum* alone produced any regressions. *C. parvum* prior to cyclophosphamide was without effect, and simultaneous administrations were toxic. The time interval, 12 days, between cyclophosphamide and *C. parvum* was found to be extremely critical, 6 or 16 days producing no regressions.[60] In a similar tumor system the results have been more variable, but are still in keeping with these findings.[48] A murine leukemia has been successfully treated with 76% complete remissions using a combination of 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) followed 3 days later by intradermal *C. granulosum.*[64] Again, chemotherapy or *C. granulosum* alone was considerably less effective, and the 3-day time interval between chemotherapy and immunotherapy was found to be critical. It would seem from these two studies that the time interval may depend on the particular chemotherapy employed. Simultaneous administration of procarbazine i.p. and *C. parvum* i.v. 10 days after injection of leukemia cells fully protected all mice, whereas with procarbazine alone there were no survivors.[65,66]

No therapy data are available on combination of *C. parvum* with radiotherapy; however, a pretreatment mouse model shows that the protective effects of i.p. *C. granulosum* are resistant to subsequent low-dose (600 R) whole-body irradiation. Irradiation within 2 days before stimulation abrogated the protective effect.[67]

Another therapeutic approach has been the combination of immunostimulation with specific active immunization against the tumor using inactivated tumor cells. Biweekly systemic injections of heat- or formal-killed tumor cells plus *C. parvum* were found to be no more effective at inhibiting an ongoing solid mammary adenocarcinoma than were i.p. *C. parvum* injections alone.[53] Woodruff and Dunbar found that simultaneous subcutaneous injection of irradiated neuraminidase-treated tumor cells and i.p. *C. parvum* may cause a more prolonged remission than either treatment alone.[48] The author's current studies (in preparation) using a mouse mastocytoma model show that s.c. injection of irradiated tumor cells mixed with *C. parvum* confers strong specific immunity against both subcutaneous and i.v. tumor challenge. This immunity is also detectable as transient tumor growth inhibition in a therapy model.

The combination of surgical excision of the primary tumor and either *C. parvum* or *C. parvum* plus irradiated tumor cells has been studied using a rat hepatoma model.[68] It was found that immunization with a combination of intradermal and i.p. *C. parvum* prior to excision of the primary tumor had no effect on the subsequent development of lung metastases. Mixtures of *C. parvum* and irradiated cells had an overall therapeutic effect, reversing the facilitating effect found with irradiated cells alone. If therapy was delayed until after excision of the primary tumor, *C. parvum* alone, as well as in combination with irradiated cells, reduced both the incidence and number of lung metastases.

A synergistic antitumor effect between *C. parvum* stimulation and tumor cells that had been preincubated in vitro with heterospecific antitumor globulin has been described;[51,69] however, the failure to demonstrate any effect in vivo[51] would seem to eliminate this approach.

CLINICAL DATA

Few definitive clinical data concerning *C. parvum* have been published. Reports thus far deal only with the use of *C. parvum* in simultaneous combination with chemotherapy.[70,71] Clinical trials began in France under Professor Israel in 1967 with a study of patients with advanced metastatic visceral cancers. Because of possible adverse effects of *C. parvum,* it was decided not to treat good-risk patients nor to refuse poor-risk patients the possible benefits of chemotherapy. One group of patients received chemotherapy given as a mixture of Cytoxan (15 mg/kg), 5-fluorouracil (15 mg/kg), methotrexate (0.5 mg/kg), vinblastine (0.1 mg/kg), and rufocromomycin (0.003 mg/kg) twice a month. A second group received the same chemotherapy but in addition received a subcutaneous injection of 1 ml *C. parvum* (2 mg) in each arm once a week. To counteract local pain at the injection site, the *C. parvum* was mixed with either 2% xylocaine[70] or 1% lignocaine.[71] In both groups therapy was continued throughout the lifetime of the patient, except for discontinuation of chemotherapy during periods of low white cell or platelet counts. Subcutaneous *C. parvum* is apparently tolerated well without allergic reactions, many patients having now received weekly injections for more than 3 yr without adverse effects. Local pain and swelling are occasionally encountered, and thermic reactions up to 39°C with chills lasting many hours are reported to have occurred.[71]

In the trials the only parameters compared between the groups were survival times from the beginning of treatment. A preliminary report[70] shows that of 141 patients with various metastatic visceral cancers divided into two randomized groups, those treated with chemotherapy alone survived a significantly shorter time (mean survival time = 6.1 mo) than those receiving combination therapy (10.5 mo). Similar data were later presented for various subgroups of human cancers.[71] The mean survival times of "treated" and control groups with disseminated carcinoma of epidermoid bronchogenic origin were 9.8 mo and 5.6 mo respectively. The comparable mean survival times for oat-cell bronchogenic carcinoma were 9.1 mo and 5.0 mo. In both cases there was also an apparent difference in lesion size between the two groups. Significant differences in survival data at 12 mo are also documented for patients with advanced lymphoid sarcomas and breast carcinomas. Preliminary data for advanced melanoma with visceral metastases show twice as many survivors at 12 mo if *C. parvum* is combined with the chemotherapy.[71]

Patients with good cell-mediated immune status, as judged by positive reactivity to a range of skin-test antigens, responded better to *C. parvum* treatment than did those of poor status. There was also an apparent overall trend of conversion to positivity during the course of *C. parvum* treatment.[71] This correlation of *C. parvum* activity with the patient's immune status is interesting in light of the idea that at least some of the antitumor effects of *C. parvum* have an immune basis. This concept is discussed under Modes of Antitumor Action below.

Another interesting observation arising from the *C. parvum* clinical studies is that the combination of *C. parvum* with chemotherapy increases the hematopoietic tolerance to the chemotherapy, the number of chemotherapeutic interruptions due to low white cell or platelet counts being twice as great in the control groups.[71]

From the animal data it is apparent that the reported clinical studies have been crude in their approach, their empirical nature having been dictated by ethics and currently available knowledge. The results are encouraging in that they indicate that repeated local injections of *C. parvum* are apparently well tolerated in humans and are capable of enhancing the therapeutic effect of chemotherapy against massive tumor burdens. The optimal schedule and doses for such combination therapy remain to be determined: subsequent animal experiments[60,64] have stressed the critical importance of the timing between chemotherapy and immunostimulation, and it may well be that sequential courses of chemotherapy and immunostimulation will be more advantageous than the simultaneous therapy described.

MODES OF ANTITUMOR ACTION

Nonspecific Activity

Various results indicate that systemically administered *C. parvum* may be largely nonspecific in its action, i.e., not amplifying a specific antitumor response. Its overall effects are not abrogated in T-cell-deprived mice,[52,55] and are also resistant to antilymphocyte serum.[72,73] Any modification of tumor immunity by *C. parvum* would be expected to be apparent following combined

administration of *C. parvum* and inactivated tumor cells; however, injection of i.v. *C. parvum* 1 day after a subcutaneous injection of irradiated mastocytoma cells afforded only as much protection against tumor challenge as did *C. parvum* injection alone.[55] Also, in a therapy model, biweekly injections of i.p. *C. parvum* alone have been shown to be as effective as similar injections of mixtures of *C. parvum* with killed tumor cells.[53] The cumulative evidence of suppression of T-cell activity following systemic *C. parvum* seems to argue against boosted cell-mediated immunity determining increased tumor resistance. That antibody may not be involved is suggested by the efficacy of *C. parvum* against tumors in mice selected for low antibody responses,[74] and also by the fact that splenectomy is without effect.[73]

The nonspecific antitumor activity of *C. parvum* is most probably mediated by the *C. parvum*-activated macrophage. Peritoneal and lung macrophages from mice stimulated systemically with *C. parvum* have been shown to inhibit nonspecifically the in vitro proliferation of tumor cells.[6,55] Similar activity has been described for macrophages activated nonspecifically by other means, e.g., BCG or *Toxoplasma* infection, where the mechanism is apparently nonphagocytic and involves the secretion of lysosomal enzymes directly into the cytoplasm of the target tumor cell.[75] It is likely that the same mechanism is operative following *C. parvum* activation, and the recent findings of Wilkinson et al. are therefore extremely interesting. They demonstrated that many strains of *C. parvum* produce a chemotactic factor that specifically attracts macrophages,[76] and they further implicate this factor in the activation of lysosomal enzymes in these macrophages.[7] That such macrophage antitumor activity is operative in vivo is supported by the finding that the effects of systemic *C. parvum* pretreatment against simulated metastases are radioresistant—a characteristic of macrophages.

Specific Activity

In addition to the macrophage-mediated nonspecific antitumor mechanism, *C. parvum* is capable of boosting specific antitumor responses. The clinical data describing the conversion of skin-test reactivity in cancer patients undergoing *C. parvum* therapy[71] are indicative of augmented or restored cell-mediated immunity by *C. parvum*. In vitro evidence for elevated cell-mediated immunity has been presented by Halpern et al.[35] Using a mouse leukemia model they report that lymph node cells from mice that had received an s.c. injection of tumor cells were only minimally cytolytic for tumor cells in vitro; however, the cytolytic activity of lymph node cells from animals that also received an i.p. injection of *C. parvum* was both enhanced and prolonged. Current work in this laboratory (in preparation) shows that subcutaneous injection of *C. parvum* mixed with irradiated mouse mastocytoma cells results in strong specific systemic immunity to subsequent tumor challenge at a distant site. Irradiated tumor cells or *C. parvum* injected alone are without effect. Cell transfer studies indicate that the tumor immunity is cell-mediated. Thus *C. parvum* is capable of boosting, or in the case of the mastocytoma apparently initiating, immune responses to specific tumor antigens. The failure of this to occur with systemic *C. parvum* treatment of solid tumors would suggest that development of immunity depends on the proximity of the tumor cells to the *C. parvum*. Current experiments, again using the mastocytoma model, show that the *C. parvum*-tumor-cell interaction required for immunity does not have to occur at the same site; it may also be mediated by a common draining lymph node. It might be presumed that systemic administration of *C. parvum* and tumor cells would result in a similar development of immunity, and the in vitro experiments cited above[35] support this presumption. But a report describing that the therapeutic tumor-suppressive properties of multiple i.p. injections of *C. parvum* alone were just as effective as similar injections of *C. parvum* plus inactivated tumor cells[53] suggests this may not always be the case.

The antitumor effects of an intralesional injection of *C. parvum* are reduced in mice that have been rendered specifically unable to mount an immune response to *C. parvum*.[61] It is likely that the antitumor activity of an intralesional *C. parvum* injection is effected by both local nonspecific macrophage activation and augmented specific tumor immunity. Thus either or both of these activities may be triggered in part by "fallout" from the specific immune response to *C. parvum*. The same phenomenon is known to occur with intralesional injections of either BCG[77,78] or *Listeria monocytogenes*,[79] and such a mechanism would certainly explain the better clinical performance of *C. parvum* in patients with better immune status.[70,71]

CONSIDERATIONS FOR FUTURE IMMUNOTHERAPY

The accumulated animal data are impressive, and the initial human data are encouraging for the consideration of *C. parvum* as a potential clinical therapeutic anticancer agent. Its ultimate evaluation must await the results of the increasing number of clinical investigations that are now either underway or porposed. In this final section I shall venture to consider some of the clinical implications of the available data.

As with any form of immunotherapy, the effects of *C. parvum* are likely to be more pronounced against minimal amounts of tumor. Clinically these situations are currently represented by reasonably good-risk patients with minimal residual disease achieved by previous surgical therapy, radiotherapy, or chemotherapy. These cases would not require the continuous maintenance therapy typical of advanced cancers, and thus the effects of *C. parvum* would be more readily evaluated. Considering our current knowledge of the mode of action of *C. parvum,* it is not only the reduced tumor load that may contribute to an expected increased effectiveness of *C. parvum* in such cases. The later stages of cancer are often accompanied by a reduced competence of the patient's immune system, and it is presumably the nonspecific component of *C. parvum* that is primarily effective in such cases. Other components of the antitumor activity of *C. parvum* involves immunity to both *C. parvum* and the tumor, and these would be expected to be facilitated by a maximum degree of patient immunocompetence.

Various negative considerations concerning the injection of *C. parvum* systemically have accumulated from the animal data. Toxicity in animals has been encountered, especially with the high doses that seem to be required to effect maximum antitumor activity against solid tumors. If, as indicated, specific antitumor immunity may not result from the use of systemic *C. parvum* injections against solid tumors, this might prove to be critical in cases of possible "escapes" following only partially successful treatment. Relatively little is known, however, about the effects of systemic *C. parvum* in therapy situations against "systemic" tumors, e.g., leukemias. Also, i.v. *C. parvum* would be expected to localize in the liver and lungs and may prove to be highly effective against tumors or metastases in these organs and result in systemic immunity. One further consideration is that the phenomenon of depressed cell-mediated immunity following *C. parvum* may be peculiar to the systemic route of injection.[30]

Local injection of *C. parvum,* subcutaneous or intradermal, is not toxic and may be highly effective if used strategically. A situation in which it may be expected to have a pronounced effect is when injected directly into the tumor lesion, as for example with cutaneous neoplasms and other readily accessible lesions. It should be noted that too high a dose of *C. parvum* may "overstimulate" draining lymph nodes and impair their efficiency.[61] If intralesional injection is not practical, the injection as near to the lesion as is practical (certainly, if possible, engaging the tumor-draining lymph node) should be advantageous. In the case of widely disseminated disease, it follows that injections of *C. parvum* at many different sites, rather than restricting *C. parvum* to a single site, may be more effective. If conditions necessitate a local injection of *C. parvum* at a site distant from the tumor, then a case is made for mixing inactivated tumor cells with *C. parvum.* This would seem at least to assure the augmentation or development of systemic antitumor immunity. Any such procedure that augments the immune response to tumor antigens is open to the risk of selectively boosting the "wrong" component of the response, in this case blocking antibodies. On this point, thus far, the author is unaware of any published reports of enhanced tumor growth following therapy with either *C. parvum* alone or *C. parvum* combined with either inactivated tumor cells or chemotherapy. *C. parvum* in combination with irradiated rat tumor cells is reported to reverse the facilitating effect that large doses of irradiated tumor cells alone have on tumor growth.[68] A report that in a pretreatment model i.v. *C. parvum* prior to i.p. injection of irradiated leukemia cells compromized the protective effect of irradiated cells alone is not compatible with blocking antibody,[80] but is in keeping with the depression of cell-mediated immunity known to result from i.v. *C. parvum* prior to antigen,[30] as discussed above. These results are encouraging, but the possibility of tumor enhancement under certain conditions cannot be ruled out.

The importance of timing in any therapeutic approach involving combination of *C. parvum* and chemotherapy has been stressed and elaborated upon in both the animal- and clinical-data sections.

A point from the animal data that deserves reiteration is that these parameters may well be different for different cytostatic agents. Mathé has reported that in both animals and humans the intensity of chemotherapy, whether administered as maintenance or preimmunotherapy, influences the effect of subsequent immunotherapy.[81] Animal and human data concur, however, that chemotherapy is certainly not incompatible with immunostimulation using *C. parvum,* and given the right conditions the two antitumor effects may synergize. No similar data are available for combination of irradiation with *C. parvum* in therapy situations. The effects of systemic *C. parvum* in mice are resistant to the effects of whole-body low-dose irradiation (600 rads), but irradiation shortly before *C. parvum* administration abrogated its effect.[67] This latter phenomenon may be expected to fade if *C. parvum* administration is delayed sufficiently, and the situation may even be analogous to that described for combination with chemotherapy where a critical time interval for potentiation of the *C. parvum* effect exists. It has been reasoned that the effects of systemic *C. parvum* may be largely nonspecific and macrophage-mediated, in which case the radioresistance of the C. parvum effect is not surprising. That certain administrations of *C. parvum* do involve specific antitumor immune responses complicates the picture, since these would be expected to be relatively sensitive to subsequent whole-body irradiation.

Attention to the use of bacterial vaccines as nonspecific stimulants in cancer therapy has been largely focused on BCG, with a resultant relative wealth of experimental and clinical data. It is apparent that BCG and *C. parvum* may be similar with respect to their modes of antitumor activity. Macrophages activated by either vaccine demonstrate nonspecific antitumor activity in vitro; in addition, in vivo antitumor effects of both apparently result in part from the "fallout" from an immune response to the organisms themselves. Again, augmentation of specific antitumor immune responses is common to the ways in which their antitumor activities are effected. There is a practical difference between the two organisms; whereas *C. parvum* is fully effective when administered in the form of a killed vaccine, killed BCG is apparently ineffective.[77] The requirement for live BCG subjects it to variability arising from loss of viability during storage or transportation and further allows only a retrospective knowledge of the actual dose administered. *C. parvum* is readily administered in standard doses with no such problems of deterioration and, more important, no danger of any resulting systemic infection. These practical advantages of *C. parvum* are only relevant should the two vaccines prove to be equally effective in given situations. No valid comparative data as to this point are available, studies thus far having lacked experimental compensation for dose, timing, and other discrepancies arising from the requirement of living BCG. Subject to these qualifications, *C. parvum* has been described to be as effective as BCG in reducing rat lung metastases,[68] considerably more effective against a mouse mammary carcinoma[48] and hamster melanoma,[63] and less effective against two mouse leukemias.[56,66] Comprehensive comparative studies may reveal differences in modes of action or patterns of activity that are sufficient to form the basis for possible future combination therapy using both vaccines.

Data are now accumulating on the antitumor activity of various fractions of BCG, which may be as effective as viable organisms.[82,83] It is expected that material fractionated from *C. parvum* will become available, and it will be interesting to see the relationship between the nature and activity of both the *C. parvum* and BCG antitumor fractions.

It is evident from the animal studies that tumors seem to vary in their response to treatment with *C. parvum*; however, no characteristics related to the various susceptibilities are thus far apparent. One report correlated the response with the relative immunogenicity of the tumors,[80] but consideration of subsequent work suggests the situation to be more complex—some poorly immunogenic tumors responding well to treatment. Mathé has noticed that variations in the durations of remission and of survival of human leukemias induced by chemoimmunotherapy are related to the cytological variety of the leukemia.[81] Stiffel et al.[2] have shown that the degrees of stimulation induced by both *C. parvum* and BCG are different for inbred strains of mice; thus, genetic constitution may be another intrinsic source of variability in any future protocol using these agents.

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ANTITUMOR ACTIVITY OF *Corynebacterium parvum*

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I. Introduction

Interest in the bacterium *Corynebacterium parvum* (CP) as an immunostimulant was first aroused after the demonstration by Halpern and colleagues (1964) that injection of killed organisms into mice caused intense stimulation of the lymphoreticuloendothelial system. This was at a time when other biological reticuloendothelial stimulants, e.g., zymosan (yeast cell walls) and Bacillus Calmette-Guérin (BCG) organisms were being found to be capable of inhibiting the growth of transplantable tumors in mice (Old *et al.*, 1960, 1961). CP was

shown to be similarly effective in 1966 (Halpern *et al.*, 1966; Woodruff and Boak, 1966), and preliminary clinical trials were initiated in 1967 (Israel and Halpern, 1972).

The antitumor properties of CP in animals and preliminary clinical data were reviewed by Scott in 1974 (1974d), and the amount of data, concerning both its mode of action in animals and clinical experiences, that have accumulated during the past 2 years reflects the current level of interest in CP as a potential immunotherapeutic anticancer agent. This review considers first the immunological modifications resulting from CP administered in non-tumor-bearing animals. Animal tumor data and finally the clinical data are then considered. Throughout the reported studies the bacteria used have been in the form of either heat- or formalin-killed vaccines.

II. Terminology and Definitions

A number of anaerobic coryneforms known by the general designation *Corynebacterium parvum* possess lymphoreticular stimulating activity (Adlam and Scott, 1973; O'Neill *et al.*, 1973; McBride *et al.*, 1975a). However, until recently the taxonomic classification of these organisms has been confused and, in some instances, it has been difficult to establish the taxonomic identity or relationship between the different organisms used by various workers. Throughout this review the taxonomic descriptions of the original authors have been retained, this most often being CP. An alternative strain that has figured prominently in animal tumor studies is *Corynebacterium granulosum* (CG).

Johnson and Cummins (1972) and Cummins and Johnson (1974) have attempted to clarify the taxonomy of the anaerobic coryneforms using serology, cell wall composition, and DNA homology. Their conclusions were that they should be called Propionibacteria, since they produce propionic acid by fermentation, and should be divided into three groups—*P. acnes*, *P. granulosum*, and *P. avidum*. According to this scheme, the Burroughs Wellcome *C. parvum* vaccine strain 6134 is classified as *P. acnes* type I (C. Adlam, personal communication), and the Institut Merieux vaccine IM 1585 as *P. avidum* (Roumiantzeff *et al.*, 1975b). It is apparent that another group of anaerobic coryneforms, isolated mainly from dairy products and referred to as the "classical" Propionibacteria of van Niel (1928) are different from *P. acnes*, *P. granulosum*, and *P. avidum* (Johnson and Cummins, 1972). The "classical" Propionibacteria have thus far been uniformly negative in stimulatory activity (O'Neill *et al.*, 1973; McBride *et al.*, 1975a).

There have been studies to determine whether lymphoreticular

stimulatory activity correlate with particular species. O'Neill *et al.* (1973) found activity among representative organisms from *P. acnes*, *P. granulosum*, and *P. avidum* with no single species showing outright superiority. McBride *et al.* (1975a) confirmed activity in organisms belonging to *P. acnes* and *P. avidum* and described a soluble cross-reacting antigen produced only by these strains.

III. Stimulation of the Lymphoreticuloendothelial System

A. Changes in Organ Weight and Histology

A single intravenous injection of CP into mice produces proliferation of lymphohistiocytic elements in the liver and spleen, resulting in a marked enlargement of these organs. The increase in organ weight is evident within a few days of CP injection, peaks in the second week, and then gradually returns to normal (Halpern *et al.*, 1964; Adlam and Scott, 1973). The degree of splenomegaly and hepatomegaly depends directly on the amount of CP injected, but the kinetics of the responses are relatively dose-independent (Adlam and Scott, 1973). Repeated injections provoke more intense, longer-lasting reactions (Halpern *et al.*, 1964).

In addition to the splenomegaly (Halpern *et al.*, 1964; Howard *et al.*, 1967; Adlam and Scott, 1973; O'Neill *et al.*, 1973; Woodruff and Dunbar, 1973; Bomford and Olivotto, 1974; Castro, 1974a; McBride *et al.*, 1974; Milas *et al.*, 1974a, 1975c) and hepatomegaly (Halpern *et al.*, 1964; Adlam and Scott, 1973; O'Neill *et al.*, 1973; Bomford and Olivotto, 1974; McBride *et al.*, 1974; Milas *et al.*, 1974a) that result from systemic injection of CP into mice, lung (Adlam and Scott, 1973) and lymph node (Castro, 1974a; McBride *et al*, 1974; Milas *et al.*, 1975c) weights are also increased. High doses of systemic CP may lead to thymic atrophy (Castro, 1974a; McBride *et al.*, 1974). The changes in spleen and liver weights following single subcutaneous injections of CP are minimal, but there is marked proliferation in, and enlargement of, the lymph node draining the injection site (Scott, 1974c; Tuttle and North, 1975).

Similar organ weight changes have been reported for other species: splenomegaly and hepatomegaly in the rat (Stiffel *et al.*, 1966; Brozovic *et al.*, 1975), rabbit (Pinckard *et al.*, 1968), and guinea pig (Stiffel *et al.*, 1966), and lung weight increase and splenomegaly in the rabbit (Collet, 1971) and an amphibian (Turner *et al.*, 1974), respectively.

Histologic changes in the spleen have commonly consisted of extensive proliferation of histiocytes, macrophages, lymphocytes, and hematopoietic cells: erythroid and myeloid cells and megakaryocytes (Halpern *et al.*, 1964; Collet, 1971; Brozovic *et al.*, 1975; Milas *et al.*, 1975c). Proliferation occurred predominantly in the red pulp, whereas the white pulp of mice treated with CG has been reported to undergo lymphocyte depletion (Milas *et al.*, 1975c).

Extensive proliferation of lymphocytes and histiocytes was found in the lymph nodes (Collet, 1971; O'Neill *et al.*, 1973; Scott, 1974c; Tuttle and North, 1975) and in the lung (Collet, 1971; Pinckard *et al.*, 1968).

Hepatomegaly is usually associated with mononuclear cell infiltrates (Halpern *et al.*, 1964; Milas *et al.*, 1974a; Brozovic *et al.*, 1975). The cells are predominantly histiocytes, and they form granulomas or diffusely infiltrate liver parenchyma (Milas *et al.*, 1974a). CP induces proliferation of liver macrophages and also increases the influx of extrahepatic macrophages (Warr and Šljivić, 1974c).

B. Hematopoietic Influence

CP increases hematopoietic activity in the spleen. In addition to the histological evidence already mentioned, an increased number of colony-forming units (CFU) has been demonstrated. A similar increase in CFU occurred in the bone marrow, although the total number of nucleated cells remained unchanged or was slightly reduced (Toujas *et al.*, 1972, 1974, 1975). CP has also increased the number of CFU in the peripheral blood of mice (Bašić *et al.*, 1975b).

Shortly after administration of CP, a transient leukopenia, affecting either lymphocytes alone (Milas *et al.*, 1975c; Brozovic *et al.*, 1975) or both lymphocytes and polymorphs (Woodruff and Dunbar, 1973), was found in the peripheral blood of mice. This may be related to the observation by Castro (1974a) that CP caused an absolute decrease in the number of Θ-positive lymphocytes in the thymus, and a reduced percentage in the spleen and lymph nodes. The reason for this reduction in T cells is not known; a direct effect of CP on lymphocytes, or an increased secretion of adrenal steroids due to stress have been suggested (Castro, 1974a).

Anemia may follow CP injection (Halpern and Fray, 1969; McCracken *et al.*, 1971; Cox and Keast, 1974; McBride *et al.*, 1974) and is accompanied by increased erythropoiesis in the spleen and a rise in reticulocyte count (Brozovic *et al.*, 1975). The basis for this anemia is considered to be the enhanced phagocytosis and destruction of eryth-

rocytes by the CP-stimulated reticuloendothelial system (Cox and Keast, 1974; McBride *et al.*, 1974). Soluble antigens from CP become attached to red cells *in vitro*, and any such attachment occuring *in vivo* would be expected to facilitate the phagocytic removal of the cells by virtue of their becoming opsonized by the anti-CP antibodies present in CP-treated mice (Cox and Keast, 1974; McBride *et al.*, 1974). An autoantibody directed against syngeneic red cell antigens has also been found after CP treatment (McCracken *et al.*, 1971).

C. Macrophage Activation

More macrophage colonies are formed from bone marrow cells from mice which received CP either subcutaneously (Wolmark and Fisher, 1974; Wolmark *et al.*, 1974) or systemically (Dimitrov *et al.*, 1975a; Baum and Breese, 1976). CP increases not only the number of macrophage precursors, but also their rate of proliferation (Baum and Breese 1976). The number of peritoneal (Bašić *et al.*, 1974; Yuhas and Ullrich, 1976) and alveolar (Collet, 1971) macrophages is also significantly increased after CP.

CP-activated macrophages show more rapid adherence to glass *in vitro* and increased spreading and vacuolation compared with normal macrophages (Olivotto and Bomford, 1974). Electron microscope studies have shown modifications in surface structure and distribution of lysosomes (Puvion *et al.*, 1976). Cytochemical analyses have detected increased (Wilkinson *et al.*, 1973a,b; McBride *et al.*, 1974) or modified (Puvion *et al.*, 1976) lysozomal enzyme activity and activation of phospholipase A (Munder and Modelell, 1974).

A further characteristic of the CP-activated macrophage is its acquired ability to kill, or inhibit the growth of, malignant cells *in vitro*, and this process will be described in detail in Section VII,G.

D. Phagocytic Activity

The increased phagocytic capabilities of CP-activated macrophages are reflected *in vivo* by increased clearance of particulate material from the blood: colloidal carbon (Halpern *et al.*, 1964; Prévot and Van Phi, 1964; Stiffel *et al.*, 1966; Raynaud *et al.*, 1972; Adlam and Scott, 1973; O'Neill *et al.*, 1973; McBride *et al.*, 1974; Warr and Šljivić, 1974b), bovine serum albumin (BSA) (McBride *et al.*, 1974), and ^{51}Cr-labeled sheep red blood cells (SRBC) (Warr and Šljivić, 1974b). Studies by Stiffel *et al.* (1970, 1971) have shown that the degree of

phagocytic stimulation achieved with CP in mice depends on the strain of mice used.

The liver and spleen are the major organs responsible for the increased uptake of injected material (McBride *et al.*, 1974; Warr and Šljivić, 1974b), and the ability of different coryneforms to stimulate phagocytic clearance seems to correlate with their ability to cause splenomegaly and hepatomegaly (Adlam and Scott, 1973; O'Neill *et al.*, 1973). The *in vivo* antitumor activity of various strains has, however, correlated better with splenomegaly than with stimulation of phagocytic index (McBride *et al.*, 1975a).

IV. Immune Modulation

A. Humoral Immunity

CP is a potent immunogen: specific anti-CP antibodies have been detected following injection of organisms into mice (Woodruff *et al.*, 1974; Scott and Warner, 1976), rabbits (Dawes and McBride, 1975), and man (James *et al.*, 1975; Minton *et al.*, 1976).

Systemic pretreatment of mice with CP amplifies the antibody response to various systemically injected, thymus-dependent antigens: SRBC (Biozzi *et al.*, 1968; Howard *et al.*, 1973a; James *et al.*, 1974; Warr and Šljivić, 1974a), rat erythrocytes (Howard *et al.*, 1973a), keyhole limpet hemocyanin (KLH) (Wiener and Bandieri, 1975), and BSA (James *et al.*, 1974). Both IgM and IgG responses are affected. The exclusively IgM responses of mice to thymus-independent antigens—dinitrophenol hapten coupled to a levan carrier (del Guercio, 1972) and pneumococcal polysaccharide SIII (Howard *et al.*, 1973b; James *et al.*, 1974; Warr and Šljivić, 1974a)—are also boosted.

Pinckard *et al.* (1967a,b, 1968) have studied the effects of a non-reticuloendothelial stimulating strain of CP on antibody production against BSA in rabbits. They found that systemic CP pretreatment increased the magnitude of primary and secondary responses and the relative binding affinities of the antibodies (Pinckard *et al.*, 1967a,b). Tolerance induction was also blocked (Pinckard *et al.*, 1968). The "inactive" strain of CP also increased homocytotropic antibody production whereas strains of CP with reticuloendothelial stimulating activity did not (Pinckard and Halonen, 1971). In mice, "active" strains of CP have increased the relative binding affinities of anti-BSA antibodies, but only when the mice had tumors (James *et al.*, 1974). The induction of tolerance by injection of large amounts of SIII

polysaccharide was not affected by CP pretreatment (Howard *et al.*, 1973b).

Reports of adjuvant activity of CP in guinea pigs have concerned its injection mixed with antigen either in Freund's incomplete adjuvant (Neveu *et al.*, 1964) or as a water-in-oil emulsion (O'Neill *et al.*, 1973).

CP injected systemically a few days before systemic injection of optimally immunizing doses of antigen has usually resulted in increased antibody production; however, the modification of antibody responses by CP may depend on the dose of antigen, as well as the time of its administration in relation to CP. In mice immunized with a high dose of SRBC (10^8), CP given between 7 days and 1 day before antigen produced a marked increase in plaque-forming cells (PFC), days -7 and -4 having maximum effect. If the mice were immunized with 10^6 SRBC, CP given 7 days before only slightly increased PFC, and from 4 days before to 1 day after antigen, the PFC response was depressed. A similar dose-time dependence for CP adjuvant effects against SIII polysaccharide was also found (Warr and Šljivić, 1974a).

Published studies on the adjuvant activity of CP in mice have thus far been restricted to systemic injection of CP; however, subcutaneous injection of CP mixed with SRBC into the footpads of mice increased the number of IgM (direct) plaque-forming cells in the draining popliteal lymph node (R. Bomford, to be published). CP boosts antibody responses in guinea pigs when injected locally as a substitute for mycobacteria in Freund's incomplete adjuvant (Neveu *et al.*, 1964).

The stimulatory effects of CP on antibody production are most probably mediated via CP-activated macrophages. Wiener (1975) showed that macrophages from CP-treated mice were more effective than normal macrophages at promoting an *in vitro* primary response to SRBC by macrophage-depleted spleen cells. Watson and Šljivić (1976) confirmed these data and further demonstrated that antibody responses to a macrophage-independent antigen (DNP-POL) were not affected by CP. CP-activated macrophages retain large amounts of antigen on their surfaces, and this intensified presentation of antigen to lymphocytes may be a causal factor in the adjuvant activity of CP (Wiener and Bandieri, 1975). The interaction between antigen-laden macrophages and lymphocytes may be further intensified and prolonged in lymphoid organs as a result of the chronic trapping of lymphocytes that occurs in CP-treated mice (Frost and Lance, 1973).

The predominantly T-cell-dependent IgG response to SRBC in mice is amplified by CP in T-cell-deprived mice (Howard *et al.*, 1973a). This suggests that CP may be operating as a T-cell bypass mechanism, possibly through direct stimulation of B cells. Zola (1975)

found that CP was mitogenic for B lymphocytes *in vitro;* however, another organism with no *in vivo* adjuvant properties was similarly active.

B. Cellular Immunity

In addition to the anti-CP antibodies found after CP injection, mice also develop cell-mediated immunity to CP antigens. Delayed hypersensitivity (DTH) has resulted from subcutaneous injection of a wide range of CP doses (Scott, 1974c, 1976; Tuttle and North, 1975). No DTH was detected after intravenous injection of a single high dose of CP (Scott, 1974c), but multiple intravenous injections of low doses were effective (Scott and Warner, 1976).

CP treatment has been associated with depression of cell-mediated immune response to unrelated antigens. The DTH resulting from regional injections of picryl chloride or oxazolone (Asherson and Allwood, 1971; Allwood and Asherson, 1972) and SRBC (Scott, 1974a) was markedly depressed in mice pretreated systemically with CP. CP given after SRBC sensitization did not affect DTH reactivity (Scott, 1974a). Systemic CP pretreatment has also prolonged the survival of skin allografts. Skin grafts from A or C_3H mice survived several days longer when transplanted onto CBA mice which had received CP 1 day before or after grafting (Castro, 1974a). A similar prolongation of survival of BALB/c skin grafts was observed in C3Hf/Bu mice treated intravenously with CG, 2–28 days before, but not 2 or 7 days after, skin grafting. CG pretreatment did not affect the rejection time of second-set skin grafts (Milas *et al.*, 1975c). Skin grafting across the H-Y barrier (C57B1 ♂ → C57B1 ♀), Colapinto (1975) gave a more rapid rejection of skin grafts if CP was given before, or within a few days after, grafting, but prolongation occurred if CP was delayed until 10 days after grafting.

Scott (1974a) has attempted to analyze the mechanism(s) that may underlie the depressed DTH reactivity that results from a subcutaneous injection of SRBC in mice pretreated with intravenous CP. He found that anti-SRBC antibodies were not concomitantly increased, and that neither antigen sensitization at the draining lymph node level nor the subsequent loss of sensitized cells from the node was impaired by CP treatment. An increased uptake of sensitized cells by the CP-stimulated spleen was demonstrated, and DTH depression did not occur in splenectomized mice. These results were interpreted as showing that the peripheral expression of DTH was impaired in CP-stimulated mice owing to an effective depletion of sensitized cells that were trapped in the CP-stimulated spleen (Scott, 1974a).

Evidence for a further mechanism that may be involved in the CP-mediated depression of cell-mediated immunity has come from *in vitro* studies of cells from CP-stimulated mice. The performance of these cells in *in vitro* T-cell assays is impaired. Responsiveness to the T-cell mitogen phytohemagglutinin (PHA) of spleen and peripheral blood cells, but not lymph node cells, is depressed. The spleen cells are also less reactive in an *in vitro* mixed lymphocyte reaction (Scott, 1972a). The spleen cell response to the B-cell mitogen lipopolysaccharide is either unaffected by (Scott, 1972a) or less sensitive to (Kirchner *et al.*, 1975b) CP. Similar data are reported for spleen and peripheral blood cells from CG-stimulated mice by Milas *et al.* (1975c), who also showed that the PHA responsiveness of lymph node cells was augmented after very low doses of CG. The PHA reactivity of CP-stimulated spleen cells was completely restored after removal of macrophages by iron magnet techniques (Scott, 1972b; Kirchner *et al.*, 1975b), rayon adherence columns, and carageenin treatment, but not by treatment with anti-Θ serum and complement (Kirchner *et al.*, 1975b). These data suggest a nonspecific inhibition of T-lymphocyte function by CP-activated macrophages, the lack of correlation of macrophage numbers with degree of inhibition indicating a qualitative rather than quantitative change in the macrophage population (Scott, 1972b; Milas *et al.*, 1975c).

A further example of CP-mediated depressed T-cell activity has been the protection of F_1 recipient mice by intravenous CP against the lethal effects of graft versus host resulting from a subsequent injection of parental spleen cells (Biozzi *et al.*, 1965; Howard *et al.*, 1967). Karyotypic analysis revealed a strong inhibition of the proliferation of donor cells in the spleen of CP-treated recipients. The effect could not be attributed to any augmentation of an immune response against hypothetical parental antigens or to lack of space for donor cells in the host due to gross proliferation of lymphoreticular tissue (Howard *et al.*, 1967). The results do, however, seem compatible with a CP-activated macrophage-mediated inhibition of T-cell proliferation (Howard *et al.*, 1973a).

The depressive effects of CP on cell-mediated immunity have thus far been restricted to systemically injected CP. Scott (1974a) was unable to depress DTH to SRBC using subcutaneous CP, and the *in vitro* PHA response of cells from lymph nodes directly stimulated by regional injection of CP was not depressed (Scott, 1972a). Data are now accumulating that local interaction of CP with antigen may potentiate cell-mediated immunity. Subcutaneous injection of CP mixed with tumor antigens (irradiated tumor cells) results in strong specific cell-mediated antitumor immunity, whereas injection of CP or antigen

alone are without effect (Bomford, 1975; Scott, 1975b). Subcutaneous injection of CP mixed with SRBC also potentiates DTH to SRBC (M. T. Scott, unpublished data). Subcutaneous injections of CP (Neveu *et al.*, 1964) or CG (Degrand and Raynaud, 1973), when mixed with antigen in Freund's incomplete adjuvant, have produced DTH in guinea pigs.

The fact that systemic CP pretreatment augments IgG antibody response to thymus-dependent antigens (see Section IV,A) implies that the normal cooperative activity of T cells is not impaired by CP.

V. Resistance to Infection

Pretreatment of mice with CP has protected them against subsequent infection with a variety of bacteria: *Staphylococcus aureus* and *Bordetella pertussis* (Adlam *et al.*, 1972), *Brucella abortus* (Adlam *et al.*, 1972; Halpern *et al.*, 1973), *Salmonella enteritidis* (Collins, 1974; Collins and Scott, 1974), *Listeria monocytogenes* (Fauve and Hevin, 1971; Swartzberg *et al.*, 1975; Ruitenberg and van Noorle Jansen, 1975). The effects have been evident both from prolonged survival or permanent protection against infection and from decreased bacterial counts in the liver and spleen. It is apparent that the CP-induced bacterial resistance is immunologically nonspecific and mediated by the enhanced bactericidal capabilities of the CP-activated spleen and liver macrophages (Fauve and Hevin, 1971, 1974; Collins and Scott, 1974; Fauve, 1975). Protection against *Salmonella enteritidis* did not correlate with any augmentation of specific anti-*Salmonella* immunity (Collins and Scott, 1974), and protection against *Listeria monocytogenes* still occurred in the athymic nude mouse (Ruitenberg and van Noorle Jansen, 1975). CP treatment after bacterial infection has not been protective (Philippon *et al.*, 1972; Collins and Scott, 1974).

CP also protects against protozoal infections. An increased resistance to malaria was caused by CP injected intravenously 6–19 days before intravenous injection of *Plasmodium berghei* sporozoites (Nussenzweig, 1967). The mice exhibited delay in the onset of detectable infection and prolonged survival, and some were completely protected. A similar protection against *P. vinckei* and *P. chabaudi*, but not *P. berghei*, has been reported by Clark *et al.* (1977). They also found that systemic CP pretreatment of mice afforded complete and chronic protection against infection with *Babesia microti* or *B. rodhaini*. Intraerythrocytic death of both *Plasmodium* and *Babesia* was evident in these studies (Clark *et al.*, 1977). Mice injected intravenously, but not intraperitoneally, with CP have shown increased resistance to the

Tulahuen strain of *Trypanosoma cruzi*, and significant protection still occurred if CP was delayed until 7 days after injection (Kierszenbaum, 1975). A macrophage-mediated mechanism was indicated by the interesting finding that CP protected against infection only with the reticulotropic Tulahuen strain, but not with the predominantly myotropic Y strain of *T. cruzi*. CP-pretreated mice are also resistant to *Toxoplasma gondii*, and peritoneal macrophages from these mice are capable *in vitro* of killing *Toxoplasma* (Swartzberg *et al.*, 1975).

In contrast to these protective findings, Bryceson *et al.* (1972) observed that subcutaneous pretreatment of guinea pigs with CP emulsified in Freund's incomplete adjuvant increased susceptibility to cutaneous infection with *Leishmania enriettii*. Treatment with CP did not influence delayed hypersensitivity to *Leishmania*, but caused production of anti-leishmanial antibodies. The authors explained their findings by "regional antigenic competition" between CP and leishmanial antigen, which may alter the distribution and processing of leishmanial antigen or the traffic of *Leishmania*-sensitive cells within draining lymph nodes. Intravenous inoculation of CP also depressed the resistance of rats to *Trichinella spiralis*, as expressed in a delay of expulsion of adult worms from rat intestinal tract. This may be related to a depression of the T-cell-mediated immunologic defense against *Trichinella spiralis* (Ruitenberg and Steerenberg, 1973). Depression of cell-mediated immunity by CP is discussed in Section IV,B.

The effects of CP against viral infections have also been studied. Intraperitoneal and intravenous inoculations of encephalomyocarditis virus caused the death of 95% of normal Swiss mice, but less than 50% of those treated intraperitoneally with 0.5 mg of CP 2 or 7 days earlier. Smaller and larger doses of CP were less effective (Cerutti, 1975). Peritoneal exudates of CP-treated mice contained a factor that inhibited multiplication of both encephalomyocarditis virus and vesicular stomatitis virus. The inhibitor was resistant to heating at 56°C for 30 minutes, but did not require the integrity of cell-protein synthesis, a characteristic of the biological activity of interferon. CP was also incapable of inducing interferon in an *in vivo* model (Cerutti, 1975). Other studies have shown that treatment of mice with *C. acnes*, an organism closely related to CP, suppresses the level of serum interferon induced by injection of Newcastle disease virus of Chikungunya virus (Farber and Glasgow, 1972; Fischbach and Glasgow, 1975).

VI. Other Effects

Systemically injected CP (Adlam *et al.*, 1972; Adlam, 1973; Adlam and Scott, 1973) is capable of sensitizing mice to histamine, a property

previously considered to be unique for *Bordetella pertussis*. The ability of both CP and *B. pertussis* to sensitize depended on strain of mice used, strains sensitive to CP treatment being sensitive to *B. pertussis* and vice versa. Contrary to *B. pertussis*, CP-mediated histamine sensitivity was not associated with anaphylaxis. Anaphylaxis to horse serum, or ovalbumin, was not observed in mice receiving CP at various times before, after, or in combination with antigen (Adlam, 1973). The histamine-sensitizing activity of CP was not destroyed by heating or by treatment with formaldehyde. It was suggested that lysosome-rich CP-activated macrophages, because of their increased fragility, may release into the circulation large amounts of lysosomal enzymes, which then block the β-adrenergic receptors and (or) exert a direct effect on histamine levels (Adlam, 1973). A similar increase in sensitivity of CP-treated mice to endotoxin has been associated with a release of lysosomal enzymes (Howard, 1968).

Mice given high doses of intravenous CP have become lethally sensitive to normally safe doses of anesthetics, e.g., pentobarbitone and tribromoethanol, which are catabolized by the liver, but not ether (Mosedale and Smith, 1975). Using mice of a different strain, and lower doses of CP, Milas (1975) found no increased sensitivity to pentobarbitone. In rats, pretreatment with intravenous CP has increased the pentobarbitone-induced sleeping time by 100%, and this was associated with impairment of the hepatic microsomal enzyme system (Farquhar *et al.*, 1975). The anesthetic deaths reported may, therefore, be due to interference of detoxification processes in the liver by CP.

Certain strains of mice are capable of rejecting incompatible bone marrow grafts after lethal irradiation. If, however, these mice are given CP prior to irradiation, the bone marrow grafts are accepted (Cudkowicz and Bennett, 1971a,b; Lotzova and Cudkowicz, 1972; Rauchwerger *et al.*, 1976). The mode of action of CP under these circumstances is not understood.

VII. Effects on Experimental Neoplasia

A. Carcinogenesis

Baum and Baum (1974) reported that two subcutaneous injections of 0.7 mg of CP, a week apart, afforded mice protection against the carcinogenic effects of methylcholanthrene given at the time of the second CP injection. Sarcomas developed later, and their overall incidence was less than in untreated mice. Scott and Warner (1976)

injected mice once a week for 14 weeks, either intravenously or subcutaneously, with 0.035 mg of CP. Eight days after the final injection they were challenged with either methylcholanthrene or benzopyrene. The pattern of development and final incidence of benzopyrene-induced tumors for both intravenous and subcutaneous CP-treated groups was similar to the control. Methylcholanthrene induced tumors in all intravenous CP-treated and control mice, but 25% of subcutaneous CP-treated mice remained tumor free at the end of the experiment. As detailed in later sections, CP-treated mice are capable of inhibiting the growth of cells transplanted from tumors that have been chemically induced in syngeneic mice. It is therefore unclear whether the reported protective effects of CP against chemical carcinogens represent inhibition of the carcinogenic process, or merely inhibition of tumor growth once established.

There are reports that CP treatment reduces the incidence of tumors following the injection of oncogenic viruses; however, it is again unclear to what extent these results may represent neutralization of the virus, interference with the oncogenic process or inhibition of established tumor growth. In an earlier section CP was described to protect against viral infection and a virus inhibitory factor was discussed (Cerutti, 1975). CG given systemically 4 days before systemic injection of Friend leukemia virus markedly increased survival of mice (Kouznetzova *et al.*, 1974). CP also inhibits Moloney sarcoma virus (MSV) (N. H. Pazmino, M. Yuhas, and L. Milas, unpublished). Mice are sensitive to the oncogenic effects of MSV when newborn or aged. The final tumor incidence was not affected in newborn mice by CP either before or after virus inoculation, but the latent period before tumor appearance was significantly prolonged. In senescent mice, however, a 50% decrease in tumor incidence was induced by CP.

Aged DBA/2 mice have a high incidence (70%) of spontaneous mammary carcinomas. This incidence is decreased to 3–6% of the mice if, in adult life, they have rejected a subcutaneous transplant of syngeneic mammary carcinoma cells mixed with CP (Likhite, 1976). These mice are for a long time specifically resistant to tumor rechallenge and the ultimate decreased incidence of tumors may merely represent specific immunity to viral or tumor antigens.

B. Immunoprophylaxis

The first demonstration that CP pretreatment protected mice against challenge with syngeneic tumor cells was by Woodruff and Boak (1966). Intravenous CP 2 days before subcutaneous injections of either

mammary carcinoma or methylcholanthrene-induced fibrosarcoma cells delayed the appearance of tumors and prolonged survival of the mice. Using allogeneic mouse tumor systems, Halpern *et al.* (1966) showed that intravenous CP retarded growth of subcutaneously injected Betz sarcoma cells, but only minimally affected the intraperitoneal growth of Ehrlich ascites carcinoma. The latter was, however, extremely sensitive to intraperitoneal CP pretreatment.

Numerous reports have since appeared describing the protective effects of anaerobic corynebacteria against many syngeneic mouse tumors: mammary carcinomas (Woodruff and Inchley, 1971; Milas *et al.*, 1974c, 1975b), chemically induced fibrosarcomas (Fisher *et al.*, 1970; Milas and Mujagić, 1972; Bomford and Olivotto, 1974; Castro, 1974b; Milas *et al.*, 1974c; Bomford, 1975; McBride et al., 1975b), spontaneous fibrosarcomas (Smith and Scott, 1972; Bomford and Olivotto, 1974), osteosarcoma (van Putten *et al.*, 1975), chemically induced ICIGCI$_1$ tumor (Mathé *et al.*, 1973), mastocytoma (Scott, 1974b), plasmacytoma (Smith and Scott, 1972), line 1 lung carcinoma (Yuhas *et al.*, 1975), Lewis lung carcinoma (Mathé *et al.*, 1973), adenovirus 12-induced tumor (Rees and Potter, 1974), leukemias (Lamensans *et al.*, 1968; Amiel *et al.*, 1969; Smith and Scott, 1972; Mathé *et al.*, 1973; Kouznetzova *et al.*, 1974; Stiffel *et al.*, 1974; Halpern *et al.*, 1975; Roumiantzeff *et al.*, 1975a), and lymphomas (Halpern *et al.*, 1975; Roumiantzeff *et al.*, 1975a).

Methylcholanthrene-induced fibrosarcomas have been extensively studied. Systemic CP (Bomford and Olivotto, 1974; Milas *et al.*, 1974a) or CG (Milas *et al.*, 1974a,c) prevented development of artificial metastases in the lung following intravenous injection of fibrosarcoma cells. Using ^{51}Cr-labeled tumor cells, Bomford and Olivotto (1974) have shown that the injected tumor cells that settled in the lungs of the CP-treated mice were killed more quickly. Subcutaneous pretreatment with CP also induced resistance to intravenous fibrosarcoma cell challenge (Milas and Mujagić, 1972). Both subcutaneous (Fisher *et al.*, 1970) and systemic (Woodruff and Boak, 1966; Milas *et al.*, 1974a,c) pretreatments have been shown to be effective against solid tumors arising from subcutaneous fibrosarcoma cell challenge. One particular fibrosarcoma was peculiarly sensitive to intraperitoneal CG pretreatment. The time of appearance of tumors was not affected, but, when they were 6–10 mm in diameter, more than 50% regressed (Milas *et al.*, 1974c). Two comparative studies using mouse fibrosarcoma models have shown systemic pretreatment to be more effective than subcutaneous (Bomford and Olivotto, 1974; Milas *et al.*, 1974a).

The protective effects of CP pretreatment against mouse leukemias

depends on the relative routes of injection of CP and tumor cell challenge. Intraperitoneal CP pretreatment induced strong resistance against the intraperitoneal growth of AKR leukemia cells but was less effective against intravenous challenge (Lamensans *et al.*, 1968). Halpern *et al.* (1975) found that intraperitoneal CP was more effective against an intraperitoneal than an intravenous challenge with virus-induced YC8 leukemia cells. Intraperitoneal and intravenous injections are also more effective than subcutaneous or intradermal injections in protecting against intravenous L1210 leukemia (Mathé *et al.*, 1973).

Overall it is apparent that systemic injections have been superior to regional injections of CP in protecting against various tumor cell challenges, and this correlates well with the degree of lymphoreticular stimulation resulting from the two injection routes (Bomford and Olivotto, 1974; Milas *et al.*, 1974a). The role of the lymphoreticular system in CP-mediated antitumor effects is discussed in Section VII,G on modes of action. The stimulating effects of CP are relatively long lasting, and this is also true for some cases of CP-induced tumor protection. Although CP given 1–2 weeks before tumor cell challenge seems to be most effective, strong protection against AKR leukemia was still present after 35 days (Lamensans *et al.*, 1968). Good protection against fibrosarcoma cells was present at 25 days, but was lost by 50 days (Castro, 1974b) and, in another fibrosarcoma model, the effect of CG, although reduced, was still detected after 130 days (Milas *et al.*, 1975c). In contrast, the growth of poorly immunogenic line 1 lung carcinoma cells was inhibited only if injected within 10 days of CP (Yuhas and Ullrich, 1976). Smith and Scott (1972) suggested that the reduced susceptibility of different tumors to the protective effects of CP may be related to their immunogenicity. They screened several mouse tumors and found that better protection was achieved against the more immunogenic tumors.

The anatomical location of tumors may also influence their susceptibility. Yuhas and Ullrich (1976) showed that CP protected mice against subsequent intravenous or intraperitoneal injections of line 1 lung carcinoma, but not against subcutaneous or intramuscular tumor cells. Similar data have been reported for weekly immunogenic mammary carcinoma cells (Milas *et al.*, 1975b).

Although CP pretreatment usually protects better against low than high doses of tumor cells, in some instances it is relatively ineffective against very low numbers of tumor cells. Woodruff and Boak (1966) and Fisher *et al.* (1970) reported better protection against 10^4 than against 10^3 fibrosarcoma cells injected subcutaneously. Similarly 10^2

AKR leukemia cells grew better than 10^3 cells in mice pretreated with CP (Stiffel *et al.*, 1971).

C. Immunotherapy

1. *Systemic*

In the same studies in which they first described the prophylactic effects of CP in syngeneic mice, Woodruff and Boak (1966) also first described its therapeutic action. A single dose of CP injected intravenously 8 or 12 days after subcutaneous injection of syngeneic mammary adenocarcinoma cells significantly delayed the appearance of the tumors. Once palpable, however, the subsequent growth of the tumors was unaffected. In later studies an actual inhibition of the growth rate of the same mammary adenocarcinoma, and two unrelated fibrosarcomas, was apparent when CP was injected intraperitoneally 3 days after tumor establishment (Woodruff *et al.*, 1972). Intravenous CP treatment also caused a transitory, but significant, inhibition in the growth rate of a solid mastocytoma (Scott, 1974b). The growth of a solid mammary carcinoma has also been inhibited by biweekly intraperitoneal injections of CP starting either at the time of tumor injection (Likhite and Halpern, 1973) or 14 days later (Likhite and Halpern, 1974). Conflicting results have been reported for Lewis lung carcinoma. Intravenous CP given to mice within several days after subcutaneous transplantation of tumor cells either caused inhibition of tumor growth (Sadler and Castro, 1976) or had no effect (Morahan *et al.*, 1976).

In none of the above studies were any tumor regressions observed, but, using a methylcholanthrene-induced fibrosarcoma in C3Hf/Bu mice, Milas *et al.* (1974d) obtained complete regressions of subcutaneously growing tumors when CG was injected intravenously 3 or 7 days after tumor cells. This tumor grows rapidly and by 7 days was about 6 mm in diameter. The responses of individual tumors were extremely variable. Some of them regressed completely and permanently, others regressed partially and regrew, and others grew slightly more slowly than in controls (Milas *et al.*, 1974d). A characteristic of the regression of these tumors was that the tumor continued to grow at the same rate as in control mice for at least 10 days before the regression occurred. Systemic injections of CP also caused complete regressions of this tumor (Milas *et al.*, 1974a; Suit *et al.*, 1975, 1976b), its effect being equal to that of CG (Milas *et al.*, 1974a).

In keeping with the pretreatment studies (Smith and Scott, 1972), it may also be that the immunogenicity of tumors affects their suscepti-

bility to systemic CP therapy. Regressions have been achieved using intravenous CG against a poorly immunogenic mammary carcinoma, but, in contrast to a more immunogenic fibrosarcoma, they were extremely rare and only temporary (Milas *et al.*, 1975b). The efficiency of systemic CP may also depend on the site of tumor growth. Intravenous or intraperitoneal CP was more effective against a mouse fibrosarcoma growing intradermally or subcutaneously, than intramuscularly (Suit *et al.*, 1976b), and pulmonary deposits of this tumor were more susceptible than deposits that settled in the brain or heart (Milas *et al.*, 1974a).

The therapeutic effects of CP and CG against the artificial lung metastases that result from intravenous injection of fibrosarcoma cells have been extensively studied (Milas and Mujagić, 1972; Bomford and Olivotto, 1974; Milas *et al.*, 1974a; Milas and Withers, 1976). When the bacteria were injected systemically within a few days of tumor cell injection, they reduced the number of metastatic tumor nodules in the lung and completely cured many mice (Milas *et al.*, 1974a; Milas and Withers, 1976).

Variable results have been achieved with therapy of experimental leukemias using systemic CP. Intraperitoneal CP given as a single injection, or 5 times, at 4-day intervals, only minimally affected survival of mice which received subcutaneously L1210 leukemia cells 1 day prior to CP treatment (Mathé *et al.*, 1969). In another study using the same tumor, a significant effect was produced by multiple intravenous, or intraperitoneal, treatments of mice with CG; the treatment was started 3 or 4 days after tumor cell inoculation (Kouznetzova *et al.*, 1974). Systemic CP has retarded the growth of Moloney virus-induced YC 8 lymphoma (Halpern *et al.*, 1975; Roumiantzeff *et al.*, 1975a), but had no effect on Graffi leukemia (B. Halpern *et al.*, 1975).

All the above studies have used transplanted tumors. Thus far there is only one study concerning the therapeutic effects of CP on autochthonous tumors. Yuhas and Ullrich (1976) have described that three weekly intraperitoneal injections of CP caused temporary regressions of spontaneous mammary tumors growing in old, previously irradiated, mice.

Systemic CP treatment is most effective against small tumor masses, i.e., when injected soon after inoculation of tumor cells. The efficacy of both intravenous CG (Milas *et al.*, 1974d) and CP (Suit *et al.*, 1976b) treatment of C3H fibrosarcomas is substantially reduced for tumor sizes above 5–6 mm.

Doses of CP used for systemic injection have varied, but have been mostly in the range of 0.1–1 mg dry weight of organisms, the most

frequent dose being around 0.5 mg. Milas *et al.* (1974d, 1975a) used single injections of 0.25 or 0.5 mg to cause complete tumor regressions in their mouse fibrosarcoma model. Suit *et al.* (1976b) studied the dose response of the single injection of intravenous CP given 4 days after the same tumor. There was a fall in efficacy as the dose was decreased from 0.35 to 0.1 mg. Doses of 0.7 and 1 mg were no better than 0.35 mg but were more toxic. Toxicity of high doses of systemic CP has been reported by others (Currie and Bagshawe, 1970; Fisher *et al.*, 1970; Milas *et al.*, 1974c; Scott, 1974b). Scott (1974b) observed that the highest single intravenous dose of CP that could be tolerated (0.7 mg) was most effective in inhibiting the growth of solid murine mastocytoma. Multiple high doses of CP have usually not resulted in better antitumor activity (Scott, 1974b; Milas *et al.*, 1975b; Suit *et al.*, 1976b) but may be more toxic (Fisher *et al.*, 1970). In a fibrosarcoma therapy model, Suit *et al.* (1976b) have shown that multiple doses of intravenous CP, individually ineffective, were effective. The same finding has been described for a pretreatment situation (Milas *et al.*, 1975c). Multiple low doses of systemic CP have been shown to cause lymphoreticular stimulation and immunologic modifications similar to those of a single high dose (Scott and Warner, 1976), and this mode of application of CP may provide a means of avoiding toxicity in clinical situations.

Oral administration of CP has been tested and found to be ineffective in the therapy of the subcutaneously growing Lewis lung carcinoma. This route of injection also failed to cause splenomegaly (Sadler and Castro, 1975).

2. *Local*

Direct injection of CP into growing solid tumors has been a particularly effective form of therapy, complete and permanent regressions being a common observation in many different tumor models. When a murine (Likhite and Halpern, 1974) or rat (Likhite, 1974) mammary carcinoma growing for 14 and 20 days, respectively, was injected with CP they all regressed permanently. Not only did the injected tumors regress, but their metastases as well. Strong inhibition of tumor growth, or some complete regressions, following intralesional CP or CG therapy have also been described for mouse fibrosarcomas (Milas *et al.*, 1975b; Tuttle and North, 1975; Woodruff and Dunbar, 1975; Morahan *et al.*, 1976; Suit *et al.*, 1976b), a mouse mastocytoma (Scott, 1974c, 1976), and a hamster melanoma (Paslin *et al.*, 1974). Subcutaneous injections of CP at sites distant from the growing tumor only minimally affect its growth, whereas injections into the region of the

tumor cause marked inhibition of growth but are less effective than intralesional injections (Likhite and Halpern, 1974; Scott, 1974c; Woodruff and Dunbar, 1975).

Animals whose tumors have undergone complete regressions following intralesional CP therapy are specifically resistant to reinoculation of cells from the same tumor (Likhite, 1974; Scott, 1974c; Woodruff and Dunbar, 1975). Mice which have rejected tumors that develop from tumor cells admixed with CP are similarly, specifically immune (Likhite and Halpern, 1974; Likhite, 1976). The immunity is strong and long lasting (at least 20 months), and immune mice ultimately develop fewer spontaneous tumors (Likhite, 1976). The nature of this immunity is discussed in detail in Section VII,G.

Doses of intralesional CP that have been used, and found to be effective, have varied between 0.02 and 0.7 mg, however, different tumor models and sizes of tumor have been studied. Scott (1974c) investigated the optimal dose of intralesional CP for a mouse mastocytoma and found that, within the dose range 0.007–0.35 mg, 0.07 mg was optimal. The reduced effectiveness of higher doses of intralesional CP contrasted with the effects of intravenous CP against this tumor where the highest doses were found to be most effective (Scott, 1974b).

In keeping with systemic CP therapy, better results are achieved with intralesional therapy by shortening the time interval between tumor cell transplantation and CP. CP injected 6 days after tumor cell inoculation caused 4 out of 6 mouse mastocytomas to regress completely, and only 1 out of 6 after 10 days (Scott, 1974c). Similar data have been reported for a mouse fibrosarcoma (Woodruff and Dunbar, 1975).

Using the C3H fibrosarcoma model, which is extremely sensitive to systemic CP, i.e., complete regressions are achieved, Suit *et al.* (1976b) compared the efficacy of intravenous with intralesional CP therapy and found more regressions after intravenous treatment. With tumors less sensitive to systemic CP, intralesional CP has been found to be superior: mouse mammary carcinoma (Likhite and Halpern, 1974), rat mammary carcinoma (Likhite, 1974), mouse mastocytoma (Scott, 1974c), and mouse fibrosarcoma (Woodruff and Dunbar, 1975). Milas *et al.* (1976) reported the two routes to be equally effective against a mouse mammary carcinoma. Morahan *et al.* (1976) found that intravenous CP had no effect against Lewis lung carcinoma, and results with intralesional CP were variable, ranging from enhanced tumor growth to complete regressions.

The overall results with intralesional CP in animal tumor models are

impressive, but it is again apparent that some tumors are more susceptible than others. This variability may again be related to the individual immunogenicities of the tumors; a component of the local tumor destruction caused by CP is considered to be immunologically mediated (see Section VII,G). The degree of local tumor destruction would also be expected to be related to the distribution of CP within the tumor, and differences in the individual injection techniques may be another source of variation.

There have been few studies combining systemic and intratumor therapy with CP. Scott (1974c) reported that intravenous CP reduced the antitumor effects of a subsequent injection of CP against a weakly immunogenic murine mastocytoma. In contrast, Suit *et al.* (1976b) demonstrated that a relatively strong immunogenic mouse fibrosarcoma responded better to the combination of intravenous and intralesional CP, regardless of whether the intravenous inoculation preceded or followed intralesional CP, than to the individual treatments alone.

D. Combination with Specific Active Immunotherapy

The local interaction of CP with live or irradiated tumor cells results in strong, specific, long-lasting, cell-mediated tumor immunity (see Section VII,G). Specific active immunotherapy using mixtures of CP and irradiated tumor cells has been shown to inhibit or prevent the growth of mouse mastocytomas (Scott, 1975b) and mouse fibrosarcomas (Bomford, 1975), respectively. Subcutaneous injection of CP mixed with irradiated fibrosarcoma cells inhibited the growth of live tumor cells injected at a distant site 2 or 6 days previously. Therapy delayed until 10 days was ineffective. Successful therapy required a minimal number of irradiated tumor cells ($\geq 5 \times 10^4$), with no upper limit being detected, and only small doses of CP (0.0014–0.1 mg). There was an upper limit for the amount of CP in the mixtures which increased with the number of tumor cells. This suggests a necessity for a balance between the amount of CP and tumor antigen for optimal immunity. The small doses of CP that, in combination with irradiated tumor cells, completely inhibited tumor growth, contrasted with the larger amounts (0.35 mg) that were considerably less effective after intravenous injection (Bomford, 1975). Similar observations on the efficacy of very small doses (0.0035 mg) of CP injected in combination with irradiated mastocytoma cells are those of Scott (1975b).

Scott (1975b) was unable to potentiate tumor specific immunity using systemic injections of irradiated mastocytoma cells mixed with CP. The CP did, however, abolish the enhancing effect of the ir-

radiated tumor cells alone. Likhite and Halpern (1973) using a mouse mammary carcinoma and rat Shay chloroma also found that biweekly therapeutic intraperitoneal injections of CP and killed tumor cells were no more inhibitory than intraperitoneal injections of tumor cells alone. That tumor immunity may be stimulated using systemic CP injections is, however, evident from other studies. Combination of CP and heavily irradiated tumor cells, both given intraperitoneally, protected mice against viable sarcoma cells inoculated intravenously more effectively than the individual treatments (Milas *et al.*, 1975d). Yuhas *et al.* (1975) also reported that combination of intraperitoneal injection of CP and intravenous immunization with viable tumor cells inhibited the subcutaneous growth of line 1 lung carcinoma more effectively than either treatment alone. Combination of systemic CP and localized tumor antigen may also result in augmented tumor immunity. Woodruff and Dunbar (1973) report strong inhibition of the growth of a mouse fibrosarcoma using simultaneous injections of intraperitoneal CP and subcutaneous mitomycin C-treated tumor cells, which had been further treated with neuraminidase to increase their immunogenicity.

Under some circumstances combination of CP with irradiated tumor cells may depress, rather than potentiate, tumor immunity. Smith and Scott (1972) have shown that CP given intravenously to mice 7 days before intraperitoneal immunization with irradiated leukemia cells depressed the protective effects of the immunization. This may be related to the depressive effects of systemic CP pretreatment on various T-cell-mediated immune phenomena that we have already discussed.

E. Combination with Conventional Modalities of Cancer Treatment

The foregoing studies have shown that immunotherapy with CP, in keeping with immunotherapy in general, is effective only against relatively small tumor masses. Clinically, this is likely to represent a minimal disease situation, i.e., when the primary tumor burden has been reduced by more conventional forms of therapy—radiotherapy, chemotherapy, or surgery. This section describes the animal data concerning combination of CP with these treatments.

1. *Combination with Radiotherapy*

Tumor response to radiotherapy depends not only on radiobiological factors (Withers, 1974), but also on the immune competence of the

tumor hosts (for more details, see Milas *et al.*, 1975a). Both CP and CG have been combined with local irradiation in the therapy of experimental tumors (Milas *et al.*, 1975a,e; Suit *et al.*, 1975, 1976a,b; Moroson and Schechter, 1976). Intravenous CP (0.1–0.5 mg) reduced the radiation dose necessary to achieve local control of a relatively strongly immunogenic 8-mm mouse fibrosarcoma in 50% of treated animals (TCD 50 values) (Milas *et al.*, 1975a; Suit *et al.*, 1976b). Tumors were irradiated with single doses of γ-irradiation ranging from 200 to 4400 rads. CP also significantly augmented radiocurability of fractionated irradiation: 500 rads given for 3, 6, or 10 consecutive days (Milas *et al.*, 1975e) or 10 equal doses ranging from 50 to 950 rads with 2 days between irradiations (Suit *et al.*, 1976b). Tumors that were not cured by these combinations grew more slowly, and metastasized less frequently than tumors treated with irradiation alone. An interesting observation was that CP greatly augmented radiocurability of doses between 200 and 2500 rads whereas little improvement occurred when tumors were being controlled with about 80% probability. The TCD_{50} value for this tumor in normal mice is approximately 3400 rads. Higher irradiation doses significantly injure stromal tissues including blood vessels, and this may result in inaccessibility of surviving tumor cells to the immune defense mechanisms. High irradiation doses may also inactivate local lymphocytes or macrophages.

Suit *et al.* (1976b) studied the effects of several routes of CP administration, dose levels of CP, and time relationships between treatments of CP and irradiation. Intravenous CP regularly potentiated radiocurability. Intralesional and subcutaneous CP were, however, more effective against tumors if applied alone. When CP was given intralesionally and intravenously when tumors were 5 and 8 mm, respectively, followed by 200 rads local irradiation, the effect was less than with CP alone. In the reverse situation, i.e., first CP intravenously and then intralesionally, augmentation of radiocurability was observed. Single intravenous doses of CP smaller than 0.1 mg were ineffective. The sequence of CP and irradiation was also important. CP given to mice 2–4 days before irradiation was more effective than when given 2 days after irradiation.

CP is not as effective in augmenting radiocurability of weakly immunogenic murine tumors. In studies by Suit *et al.* (1976a) the effects of the combined treatment were barely evident against a mammary carcinoma, but the TCD_{50} values for two squamous cell carcinomas were significantly reduced. Local irradiation of a highly metastasizing mammary carcinoma with 6000 rads, single dose, caused complete regression of irradiated tumors, but, surprisingly, greatly increased the

number of metastases in the lung compared to that in mice whose tumors were surgically removed (Milas *et al.*, 1976). Treatment with CP before, but not after, irradiation not only prevented this effect, but reduced the number of metastases below that in amputated mice.

Under certain circumstances radiotherapy may be immunosuppressive and enhance tumor spread (Stjernswärd *et al.*, 1972). CP given before irradiation may prevent such deleterious effects. Milas *et al.* (1974b,c) found that enhancement of pulmonary metastases caused by whole-body irradiation of mice 1 day before intravenous injection of fibrosarcoma cells, can be prevented by CG treatment within 2 weeks prior to irradiation. CG was ineffective when given after radiation. Similar observations were reported for CP by Bomford and Olivotto (1974).

2. *Combination with Chemotherapy*

Despite the immunosuppressive nature of most cancer chemotherapy drugs, animal studies show that immunostimulation using CP can be successfully combined with drug therapy, often with additive, or even synergistic effects.

Most studies have used cyclophosphamide. Intradermal treatment of mice with CP 12 days after intraperitoneal injection of cyclophosphamide caused complete regression of a subcutaneously growing fibrosarcoma in about 70% of treated mice (Currie and Bagshawe, 1970). No complete regressions were achieved, although a significant retardation in the tumor growth was still present, when CP was injected 6 or 16 days after chemotherapy. When given before, or simultaneously with cyclophosphamide, CP was not effective but caused high toxicity. Using a similar tumor system Woodruff and Dunbar (1973) obtained results similar to those of Currie and Bagshawe (1970). In their experiments CP was given intraperitoneally and the best effect was achieved when injected 9 days after cyclophosphamide. Treatment of a murine leukemia with cyclophosphamide in combination with intraperitoneal CP was optimal when intraperitoneal CP was given 4 or 8 days after cyclophosphamide. This was when the leukemia was in remission. CP 12 days after cyclophosphamide, immediately prior to relapse, was ineffective (Pearson *et al.*, 1975). Intradermal injections of CG, either 3 or 6 days after cyclophosphamide treatment of a rat leukemia has been described to be no more effective than drug treatment alone (Pearson *et al.*, 1974a). The unpublished results of one of the authors (M. T. S.) are that the most effective treatment of a subcutaneously growing mouse fibrosarcoma occurs when a single dose of CP is given intravenously, or given subcutaneously mixed with ir-

radiated tumor cells, 4 days after a single intraperitoneal injection of cyclophosphamide. Fisher *et al.* (1975a,b) have studied the effects of prolonged administration of CP alone, and in combination with cyclophosphamide, on the treatment of mouse mammary carcinomas. The best effects were obtained when systemic CP was administered asynchronously at weekly intervals in combination with cyclophosphamide. The optimal time interval between cyclophosphamide injections was 7 days, and so was the interval between CP injections. Best results were obtained when the CP was given 4 days after cyclophosphamide, i.e., cyclophosphamide on days 0, 7, 14 and CP on days 4, 11, 18, etc.

There are also data describing CP in combination with other drugs. A murine leukemia has also been successfully treated, with 76% complete remissions, using a combination of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) followed 3 days later by intradermal CG: injections of drug or CG alone were considerably less effective, as was CG 6 or 12 days after BCNU (Pearson *et al.*, 1972). A different mouse leukemia responded well to a single intradermal injection of CG 3 days after BCNU, but repeated injections of CG at 3, 7, 9, and 11 days after drug were less effective (Pearson *et al.*, 1974b). Intravenous injection of CP 1 day after procarbazine fully protected all mice against leukemia cells, whereas with procarbazine alone there were no survivors (Amiel and Berardet, 1970). There is also a preliminary report that adriamycin in combination with CP is more effective in increasing the life-span of mice with leukemia than either drug or CP alone (Houchens and Gaston, 1976).

It is apparent that the combination of CP with drugs may be a promising form of cancer therapy and data concerning the principles underlying this form of therapy are clearly required: e.g., what are the drug susceptibilites of the various CP antitumor effects? The protective effect of intravenous CP against the formation of lung metastases following intravenous injection of tumor cells is still detectable in cyclophosphamide-treated mice, and CP almost entirely abolishes the enhancement of metastases by cyclophosphamide (van Putten *et al.*, 1975). Scott (1975a) has shown that an injection of cortisone acetate in CP-stimulated mice transiently inhibited the *in vitro* nonspecific antitumor activity of their macrophages and was sufficient to abolish *in vivo* resistance to a live tumor cell challenge. Studies by Fisher *et al.* (1976a) have, however, combined prolonged administration of cortisone acetate with repeated cyclophosphamide and CP injections and shown no inhibition of antitumor effects.

CP has already been described to increase the number of colony-

forming cells in the bone marrow, and a possible indirect antitumor effect in future clinical situations may be the toleration of more intensive chemotherapy due to a marrow protective effect. Further data in this area are also required, since a recent report describes that CP-stimulated mice are more susceptible to the toxic effects of high doses of 5 fluorouracil (Foster, 1976).

3. *Combination with Surgery*

No general principles underlying the combination of CP treatment with the surgical removal of solid tumors are yet apparent from the few animal data available.

Combined intradermal and intraperitoneal CP at 7 and 14 days after surgical removal of a subcutaneously growing rat hepatoma inhibited the formation of lung metastases. CP injected before amputation was ineffective. If large numbers of irradiated hepatoma cells were injected before surgery the number of lung metastases was increased, and this effect was abolished by simultaneous injection of CP (Proctor *et al.*, 1973). CP before amputation has been found to be effective in mouse models. Sadler and Castro (1976) reported that single intravenous or intraperitoneal, but not subcutaneous, injections of CP within 4 days before surgery reduced the incidence of lung metastases from a Lewis lung carcinoma. Similar data for systemic CP in a mouse mammary carcinoma model has been presented by Milas *et al.* (1976). CP or CG injected after removal of the mammary carcinoma were ineffective, and injections of CG 4 and 10 days after surgery slightly enhanced metastases.

F. Antitumor Activity of CP Fractions

It will be apparent from the clinical data (see Section VIII,B) that some of the side effects commonly observed with whole bacterial vaccines are serious. It is hoped that studies attempting to characterize the active principle of CP will result in retention of antitumor activity with a reduction in toxicity.

A phospholipid extract of CP has been reported to be less toxic than whole organisms in mice. It failed to cause splenomegaly and hepatomegaly but did inhibit the multiplication of bacteria in the liver and spleen. Its antitumor properties were not examined (Fauve and Hevin, 1974). A lipid extracted from the surface of CP has also been shown to be a chemoattractant for mouse and guinea pig macrophages and for human monocytes (Russel *et al.*, 1976). An extract of CP produced by mild hydrolysis has retained some antitumor activity, al-

though considerably less than whole organisms. A lipid component seemed to be responsible, and antitumor activity was increased if the extract was adsorbed onto latex to facilitate its phagocytosis (McBride *et al.*, 1976). Zola (1975) has shown that the *in vitro* B-cell mitogenic property of CP is lacking in a delipidated preparation of cell walls. If stimulatory activity is associated with a lipid component, then other components may be similarly active. Adlam *et al.* (1975) have shown that lipid-free cell walls of CP retained their lymphoreticular stimulating and antitumor activity against allogeneic sarcoma 180. Water-soluble extracts prepared from delipidated cell walls of CP have also been shown to stimulate carbon clearance, antibody formation and DTH, as well as protecting against encephalomyocarditis virus and MSV (Migliore-Samour *et al.*, 1974; Jollès *et al.*, 1975).

Various cell wall preparations of anaerobic corynebacteria were shown by Kouznetzova *et al.* (1974) to possess reticuloendothelial stimulating activity, adjuvant properties, and protective effects against experimental mouse leukemias. A chemical analysis of CP cell walls has been presented by Azuma *et al.* (1975), who also found that adjuvant activity for antibody formation and DTH was present in cell walls, but that cell-mediated antitumor immunity was not augmented. The adjuvant active unit of the cell wall structure is considered to be a peptidoglycan, and a similar dissociation between adjuvant and antitumor activity has been described for synthetic peptidoglycans (Azuma *et al.*, 1976; Yamamura *et al.*, 1976).

G. Modes of Action

1. *Nonspecific Activity of CP-Activated Macrophages*

The *in vivo* antitumor effects of systemically injected CP in mice have been shown to be resistant to a variety of immunosuppressive procedures, suggesting an immunologically nonspecific mechanism (i.e., not amplifying a specific antitumor response) to be operating. They are resistant to subsequent whole-body irradiation (Bomford and Olivotto, 1974; Milas *et al.*, 1974b). They are also still apparent in mice deprived of T lymphocytes by thymectomy, irradiation, and bone marrow reconstitution (TIR) (Woodruff *et al.*, 1973; Scott, 1974b) and antilymphocyte serum treatment (Hattori and Mori, 1973; Castro, 1974b). The inhibitory effects of systemic CP on various T-cell-dependent immune phenomena (discussed in Section IV,B) are also in keeping with a noninvolvement of lymphocytes in the CP-mediated antitumor response. That antibody is unlikely to be involved

is suggested by the demonstration that splenectomy, a procedure likely to impair antibody production, did not abrogate the *in vivo* antitumor effects of a subsequent injection of CP (Castro, 1974b; Mazurek *et al.*, 1976). CP effects were also apparent in mice genetically selected for low antibody responses (Biozzi *et al.*, 1972).

The resistance of systemic CP effects to immunosuppression is compatible with a macrophage-mediated response, and CP-activated macrophages exert antitumor effects *in vitro*. This is not a property unique to anaerobic corynebacteria, but is shared by many other microorganisms, such as BCG (Hibbs *et al.*, 1972; Hibbs, 1973; Cleveland *et al.*, 1974). *Toxoplasma gondii* (Hibbs *et al.*, 1972; Hibbs, 1973; Krahenbuhl and Remington, 1974), *Listeria monocytogenes* (Hibbs *et al.*, 1972; Hibbs, 1973; Krahenbuhl and Remington, 1974), and *Besnoitia jellisoni* (Krahenbuhl and Remington, 1974).

Peritoneal macrophages from mice treated with CP inhibit the *in vitro* growth and DNA synthesis of syngeneic mastocytoma cells (Scott, 1974b), radiation-induced leukemia cells (Olivotto and Bomford, 1974; Bomford and Christie, 1975; Christie and Bomford, 1975), fibrosarcoma cells (Olivotto and Bomford, 1974; Ghaffar *et al.*, 1974, 1975), and RBL-5 lymphoma cells (Kirchner *et al.*, 1975b). The ultimate fate of the target tumor cells in such cytostatic assays is not clear, but a direct cytotoxic effect of CP-activated macrophages on Lewis lung carcinoma has been described by Morahan and Kaplan (1976). Similarly, Bašić *et al.* (1974, 1975a) found that peritoneal macrophages from mice treated with CG destroy *in vitro* cultures of a syngeneic fibrosarcoma, tumorigenic mouse L-P 59 cells, transformed Chinese hamster ovary cells, and human melanoma cells.

The antitumor activity of peritoneal macrophages has been detected within 2 days following intravenous injection of CP, was maximal after 5 days, and thereafter declined but was still present at 20 days. In contrast, the antitumor activity of lung macrophages was maximal 14 days after CP (Olivotto and Bomford, 1974). The activity of splenic macrophages could be first detected 7 days after intraperitoneal CP; it peaked at 14 days and usually disappeared after 18–21 days (Kirchner *et al.*, 1975b).

It is apparent that these *in vitro* cytostatic or cytotoxic effects are exclusive to the macrophages. The effector cells used by Olivotto and Bomford (1974) were almost exclusively macrophages by both morphologic and functional criteria. Nonadherent peritoneal cells from CP-stimulated mice did not inhibit tumor cell growth *in vitro* (Ghaffar *et al.*, 1974), and procedures for removing lymphocytes from peritoneal cells, such as extensive washing, trypsinization, or irradia-

tion, did not impair their antitumor activity (Ghaffar *et al.*, 1974; Scott, 1974b; Bašić *et al.*, 1975a). Similarly, the activity of spleen cells was found to be unaffected by treatment with anti-θ-serum and complement, whereas macrophage removal using rayon adherence columns, iron-magnetic techniques, or carageenin treatment, almost entirely abolished it (Kirchner *et al.*, 1975b).

The mechanisms by which CP-activated macrophages kill, or inhibit the growth of, tumor cells *in vitro* is not clear. Medium removed from cultures of activated macrophages (Olivotto and Bomford, 1974; Bašić *et al.*, 1975a) or from cultures containing both, CP-stimulated macrophages and tumor cells (Ghaffar *et al.*, 1974) did not inhibit the growth of tumor cells *in vitro*. This suggested that the antitumor activity of activated macrophages was not mediated through substances released into the growth medium, and that macrophage-tumor cell contact may be required. After several hours of culture, adherence of macrophages to tumor cells is already apparent (Olivotto and Bomford, 1974; Bašić *et al.*, 1975a), and electron microscopy studies have shown a close apposition between the two cell surfaces (Puvion *et al.*, 1975, 1976). Adherent tumor cells subsequently undergo lysis (Bašić *et al.*, 1975a). Hibbs (1974) reported that, upon contact with target cells, macrophages activated by BCG or *Toxoplasma* appear to secrete lysosomal enzymes directly into the cytoplasm of target cells, which then lyse. This was inhibited by trypan blue, an inhibitor of lysosomal enzyme function (Hibbs, 1974). The *in vivo* antitumor effects of systemic CP are similarly sensitive to trypan blue (Morahan and Kaplan, 1976). Cortisone acetate also inhibits the *in vitro* antitumor activity of CP-activated macrophages (Scott, 1975a), the presumed effect being the prevention of exocytosis of lysosomal enzymes through stabilization of the macrophage membrane (Weissmann and Dingle, 1961; de Duve *et al.*, 1962; Allison and Davies, 1972). The lysosomal enzymes that may be involved in the *in vitro* killing of tumor cells by activated macrophages are not known. However, Puvion *et al.* (1976) recently reported no correlation between the level of acid phosphatase in CP-activated macrophages and tumor cell-killing ability.

The mechanisms of macrophage activation by CP have been the subject of recent studies by Christie and Bomford (1975) and Bomford and Christie (1975). Normal mouse peritoneal macrophages could not be activated *in vitro*, as assayed by inhibition of tumor cell proliferation, by exposure to CP alone, but only by mixtures of CP and spleen cells from CP-immune mice. Treatment of the spleen cells with anti-θ serum and complement abolished this activation. Supernatants from cultures of the CP-immune spleen cells and CP were also capable of

activating normal macrophages. This indicated an immunological pathway of macrophage activation mediated by a release of soluble factors from CP-sensitized T lymphocytes on contact with the antigen. Participation of such an immune mechanism in the *in vivo* CP activation of macrophages was evidenced by the acceleration of macrophage activation by CP in mice which had been immunized with CP 60 or 130 days earlier. Macrophages from normal mice became cytostatic 5 days after CP treatment, but those from previously sensitized mice were already cytostatic within 1 day (Bomford and Christie, 1975). It seems that CP can also activate macrophages *in vivo* by an alternative, immunologically independent pathway, since macrophage activation in both TIR mice (Bomford and Christie, 1975) and athymic nude mice (Ghaffar *et al.*, 1975) is equal to that in normal mice.

The fact that CP activates complement by the alternative pathway in both normal human and guinea pig serum (McBride *et al.*, 1975c) allows speculation as to a further pathway of macrophage activation mediated by cleavage products of complement component C3. Activation of complement by the alternative pathway generates the C3 cleavage product C3b, and Schorlemmer *et al.* (1976) report experiments in which attachment of C3b to mouse macrophages in culture results in macrophage activation as evidenced by release of lysosomal enzymes into the culture. Similarly, activated mouse macrophages have recently been shown to nonspecifically inhibit mouse tumor cell growth *in vitro* (A. C. Allison, personal communication).

The *in vitro* cytotoxicity mediated by macrophages stimulated with anaerobic corynebacteria seems to be limited to cells with malignant growth characteristics. CG-stimulated macrophages do not destroy syngeneic or allogeneic fibroblasts, or allogeneic kidney epithelial cells *in vitro* (Bašić *et al.*, 1975a). These observations accord with findings by Hibbs' group that *in vitro* cultures of normal allogeneic cells, but not cultures of cells with neoplastic properties, were resistant to destruction by murine macrophages activated with *Toxoplasma*, *Listeria monocytogenes*, or BCG (Hibbs *et al.*, 1972; Hibbs, 1973). The reasons for this discriminatory behavior of activated macrophages are not known but may be related to membrane properties of both activated macrophages and neoplastic cells facilitating, or initiating, close contact between them. Membranes of malignant and transformed cells differ from normal cells in electrical charge (Ambrose *et al.*, 1956), surface glucoprotein (Roberts *et al.*, 1973), glycolipid and glycosyltransferase activity (Hakomori and Murakami, 1968; Roth and White, 1972), and lectin agglutinability (Aub *et al.*, 1963; Burger, 1969). Activated macrophages exhibit increased sticki-

ness (Bašić *et al.*, 1975a), membrane motility (Nathan *et al.*, 1971), and membrane glycosamine incorporation (Hammond and Dvorak, 1972).

What is the evidence that a nonspecific destruction of tumor cells by CP-activated macrophages operates *in vivo*? Most of the evidence to date is indirect. The resistance of the *in vivo* antitumor effects of systemic CP to various immunosuppressive procedures, including radiation, has already been commented upon. Mouse tumors regressing following intravenous CP or CG treatment are heavily infiltrated with macrophages (Milas *et al.*, 1974a,d). It is, however possible that these cells may merely be removing the remnants of tumor cells killed by other host mechanisms. An involvement of macrophages is implied by the findings of McBride *et al.* (1975b). They showed that gold salts (sodium aurothiomalate), which inhibit macrophage lysosomal enzyme activity, suppressed the *in vivo* protective action of CP against both intravenously and subcutaneously injected mouse fibrosarcoma cells. Scott (1975a) has also reported similar suppressive effect using cortisone in a murine mastocytoma model. Current experiments in one of our laboratories (Peters *et al.*, 1977) provide more direct evidence for *in vivo* involvement of activated macrophages. Addition of peritoneal macrophages from CP-stimulated mice to fibrosarcoma cells resulted in the suppression of tumor growth when this mixture was transferred intraperitoneally or subcutaneously into syngeneic recipients. Normal peritoneal cells or heat-killed activated cells had no effect.

It should be borne in mind that these nonspecifically activated macrophages which kill tumor cells *in vitro*, and are presumed to have a similar role *in vivo*, may be the same cells that have already been described to inhibit T lymphocyte responses *in vitro* (Scott, 1972a,b; Kirchner *et al.*, 1975b). Kirchner *et al.* (1975a) have presented data that CP-activated macrophages may also be the cell type responsible for suppressing the specific secondary cytotoxic response of immune spleen cells against tumor cells *in vitro*. Systemic CP pretreatment has been described to depress the *in vivo* protective effects of specific immunization with irradiated tumor cells in an experimental leukemia model (Smith and Scott, 1972), and there thus may be a balance between the beneficial antitumor effects of CP and its immunosuppressive effects.

2. *Potentiation of Tumor-Specific Immunity*

Studies using local injections of CP have revealed a further mode of action which is immunologically specific, i.e., potentiates the host's immune response to tumor-specific antigens.

Subcutaneous injection of mixtures of CP with viable mouse mammary carcinoma or rat Shay chloroma cells into the corresponding syngeneic hosts resulted in the development of tumors that grew for about 2 weeks and then rapidly regressed. Animals whose tumors had undergone regression were found to be specifically resistant to tumor rechallenge (Likhite and Halpern, 1973). An analogous situation is the injection of CP directly into an established tumor, and animals whose tumors have undergone regression following intralesional CP therapy are similarly specifically immune to rechallenge (Likhite and Halpern, 1974; Scott, 1974c). The specific resistance resulting from local CP-tumor cell interaction is long lasting and has been detected up to 20 months after injection of CP mixed with viable mouse mammary carcinoma cells (Likhite, 1976).

The foregoing studies suggested that, under conditions of close contact between CP and tumor cells, CP may be augmenting a specific antitumor immune response. Fisher *et al.* (1974) observed that the *in vitro* cytotoxicity of tumor draining lymph node cells for mouse mammary carcinoma cells was increased after injection of CP directly into the tumor or between the tumor and the draining lymph node. Since treatment of normal mice with CP did not result in any cytotoxicity, the augmentation in tumor-bearing mice was ascribed to a CP-mediated potentiation of the specific tumor immune response. Potentiation of specific antitumor activity by CP is also evident from experiments in which subcutaneous injections of CP mixed with irradiated mastocytoma cells have specifically immunized mice against tumor rechallenge, while injections of CP or irradiated cells alone were without effect. The injection sites of CP and irradiated tumor cells did not need to be coincident but required common lymphoid drainage for immunity to occur (Scott, 1975b). Similar specific immunity arising from therapeutic injections of CP-irradiated mouse fibrosarcoma cell mixtures have been reported by Bomford (1975). It was reduced in T cell-deprived mice (Bomford, 1975; Scott, 1975b) and could be transferred by lymph node cells draining the site of mixture injection, but not by serum (Scott, 1975b). Tuttle and North (1976a,b) were also unable to transfer immunity with serum and further presented evidence that the lymphoid cell involved was a relatively short-lived T cell, which probably does not require macrophages or other lymphocytes to effect its antitumor activity. That such a cell-mediated immune mechanism may be operative in the local destruction of tumor cells following intralesional injection of CP is suggested by the complete T cell dependence of this form of CP therapy (Scott, 1974c; Woodruff and Dunbar, 1975). Tumors regressing following intralesional injection of

CP are heavily infiltrated with lymphocytes and macrophages (Likhite and Halpern, 1973, 1974).

In Section VII,G,1 it has been reasoned that the antitumor effects of systemically injected CP are predominantly nonspecific and macrophage-mediated. There are data, however, showing that potentiation of specific immunity is not exclusive to locally injected CP but may also be a component in systemic CP therapy. Halpern *et al.* (1973) reported that the *in vitro* specific cytotoxicity of lymph node cells of mice bearing leukemia was greatly augmented and prolonged by intraperitoneal treatment of mice with CP. Yuhas *et al.* (1975) observed that neither specific immunization of mice with tumor cells nor treatments with intravenous CP caused resistance to line 1 carcinoma cells, but that combination of the two was very efficient. Furthermore, CP has required the presence of T cells to induce optimal resistance to pulmonary deposits of a murine fibrosarcoma (Milas *et al.*, 1975d).

The role of antitumor antibodies in CP-induced resistance is less certain than that of immune cells. Transfer of serum from mice positively immunized by mixtures of CP and irradiated tumor cells was ineffective in conferring resistance upon normal mice (Scott, 1975b). Cytotoxic antibodies have been reported after regression of living tumor cells mixed with CP (Likhite, 1975), but these may be a result of the temporary growth of the tumor itself. In an allogeneic mouse tumor model, CP pretreatment did not modify the level of complement-dependent cytolytic antibody. It did, however, increase the antibody-dependent cellular cytotoxicity (ADCC) of spleen cells (Mantovani *et al.*, 1976). We are aware of no similar data concerning ADCC in a syngeneic tumor system. A noninvolvement of antibodies may be inferred from the undiminished protective effects of CP in splenectomized mice (Castro, 1974b; Mazurek *et al.*, 1976) and genetically low antibody producers (Biozzi *et al.*, 1972).

The mechanism(s) of the potentiating activity of CP on specific antitumor immunity is as yet unclear. The processing of tumor antigens within CP-stimulated lymphoid organs may be modified. Wiener and Bandieri (1975) have shown that macrophages from CP-treated mice retain more antigen on their surface than normal macrophages which may improve antigen presentation to lymphocytes. Another contributing factor could be the increased and prolonged lymphocyte trapping in lymph nodes of CP-stimulated mice (Frost and Lance, 1973).

3. *Immunity to CP*

In addition to its adjuvant properties, CP is itself a potent immunogen producing both agglutinating antibodies (Woodruff *et al.*, 1974;

Scott and Warner, 1976) and DTH (Scott, 1974a,c; Tuttle and North, 1975). There is evidence that the expression of an immunological response to CP antigens is important for local destruction of tumor cells following intralesional CP injection. Scott (1974c) described that the therapeutic effects of an intralesional injection of CP into an established mouse mastocytoma were reduced if the specific reactivity of the tumor-draining node to CP had been preempted by a large amount of the bacteria injected intravenously. Tuttle and North (1975) were unable to achieve regressions of a mouse fibrosarcoma following intralesional injection of CP unless the mice had been previously sensitized to show DTH to CP, and the intralesional injection was at the time of maximum CP sensitivity. The destruction of tumor cells at the site of a DTH reaction to CP was associated with the influx of phagocytic mononuclear cells. These may be activated macrophages since Christie and Bomford (1975) have shown that *in vitro* macrophages can be nonspecifically activated to kill tumor cells as a result of local interaction of CP with CP-sensitized T lymphocytes.

Immunity to CP would also be likely to influence its antitumor activity in any situation where tumor antigens might cross-react with CP antigens (James *et al.*, 1976). Another situation would be if CP antigens were to become absorbed onto the surface of tumor cells. They have been shown to be absorbed onto syngeneic red cells *in vitro* (Cox and Keast, 1974).

4. *Other Considerations*

Tumor-bearing mice contain in their circulation soluble tumor antigens and antibody–antigen complexes that may block the efficiency of immune effector cells. CP treatment may, through nonspecific stimulation of phagocytic activity, cause these factors to be removed. Examples of this may be the abolition of enhancing effects of active immunization with irradiated tumor cells reported for a rat hepatoma (Proctor *et al.*, 1973) and a murine mastocytoma (Scott, 1975b). A related observation may be the abrogation by CP of the enhanced spontaneous pulmonary metastases that resulted from local irradiation of a primary tumor (Milas *et al.*, 1976). CP treatment also alters distribution of tumor cells released into circulation (Bomford and Olivotto, 1974) and may affect the process of metastases in this manner.

Serum from mice treated with CP and several days later with endotoxin contains a substance which, upon injection into tumor-bearing mice, causes tumor necrosis (Carswell *et al.*, 1975; Green *et al.*, 1976). This tumor necrosis factor (TNF) is not produced by treatments with CP or endotoxin alone and is considered to be released by host CP-

activated macrophages on contact with endotoxin (Carswell *et al.*, 1975). Whether TNF represents a component of the *in vivo* CP-activated macrophage antitumor process remains to be determined.

Processes facilitating the accumulation of host effector cells at a tumor site would be expected to contribute to the degree of damage inflicted by immunotherapy. In this respect, two such reports seem relevant to the effects of locally injected CP. Various strains of anaerobic coryneforms, including CP, have been found to produce chemotactic factors that attract macrophages specifically (Wilkinson *et al.*, 1973b). Moore and Hall (1973) have also observed that local injections of CP caused a local change in capillaries resulting in extravasation of lymphoblasts.

Most of the data presented in this section may be considered as evidence that the CP organism is not itself toxic for tumor cells, and our own unpublished results support this. CG added to mouse sarcoma cells *in vitro* did not alter their subsequent ability to form colonies *in vitro* (L. M.), and CP added to mouse mastocytoma cells *in vitro* did not inhibit their DNA synthesis (M. T. S.).

VIII. Clinical Experience

A. Therapeutic Effects

The first preliminary study of the clinical use of CP involved 141 patients with various metastatic carcinomas who received either chemotherapy (cytoxan, 5-fluorouracil, methotrexate, vinblastine, and rufocromomycin) or a combination of chemotherapy with CP twice a month (Halpern and Israel, 1971; Israel and Halpern, 1972). Two milligrams of CP were given subcutaneously in each arm every week. Therapy was continued throughout the lifetime of patients, except when discontinued during periods of low white blood cell and thrombocyte counts. The patients were monitored only for survival, and CP was found to have a significant effect. The mean survival time was 3.8 months for patients receiving chemotherapy alone, and 8.7 months for those who received CP and chemotherapy.

Further studies by the same group (Israel, 1973, 1975; Israel and Edelstein, 1975) showed that CP could be used effectively as an adjunct to chemotherapy of lung tumors, breast carcinomas, sarcomas, and melanomas. Mean survival of patients with disseminated carcinoma of epidermoid origin under chemotherapy alone was 5.6 months; it was 9.8 months if treatment with CP was included. Simi-

larly, patients with oat-cell carcinoma survived 9.1 months if given CP and chemotherapy, but only 5.0 months if they were on chemotherapy alone. In advanced breast carcinoma, survival of patients treated with the combined modalities was 85% at 1 year and 41% at 2 years, compared with 41% and 16% of patients receiving chemotherapy alone. The 1-year survival of patients with advanced nonlymphomatous sarcomas and malignant melanomas was also significantly increased by addition of CP (Israel and Edelstein, 1975).

Immunocompetent patients, as judged by the positivity of purified protein derivative of tuberculin (PPD) skin testing, responded better to treatment with CP. An interesting observation was that CP was capable of converting negative skin tests (PPD, mumps, candidin, killed BCG, and DNCB) in about 50% of patients previously nonresponsive to these antigens.

A further observation in studies by the Israel group was that CP treatment greatly improved hematopoietic tolerance to chemotherapy. The number of chemotherapeutic interruptions due to low white blood cell and platelet counts was one-half as frequent as that in the group receiving chemotherapy alone (Israel and Edelstein, 1975), and patients treated with CP also tolerated larger doses of chemotherapy. Some recent studies have not confirmed these findings. Myelosuppression was no less severe, and the amount of chemotherapy tolerated was no greater in breast carcinoma patients who were given weekly subcutaneous injections of 4 mg of CP in addition to cyclophosphamide, adriamycin, methotrexate, and 5-fluorouracil (De Jager *et al.*, 1976). In this study CP also did not improve the effectiveness of chemotherapy. Intravenous CP (2.5 mg/m^2) also failed to reduce the myelosuppressive effects of chemotherapy in patients with breast carcinoma (Haskell *et al.*, 1976).

Subcutaneous injections of CP and CG have been used as an adjuvant to radiotherapy with ^{60}Co of patients with head and neck malignant tumors, and, in comparison with irradiation alone, slightly increased the survival rate (Mahé *et al.*, 1975). Treatment with 1mg of the bacteria commenced 6 weeks after completion of the radiation therapy. Bacteria were administered weekly for the first 6 weeks, and then once every 2 weeks for the duration of the trial.

Other preliminary reports have provided encouraging results concerning the use of subcutaneous CP in combination with surgical resection of malignant melanoma (Ishmael *et al.*, 1976) and in combination with chemotherapy of advanced ovarian cancer (Ochoa *et al.*, 1976).

Intramuscular injections of anaerobic corynebacteria have been used in immunotherapy of patients with acute lymphoid leukemia

(Schwarzenberg and Mathé, 1975). Immunotherapy was started after remission was achieved by previous chemoradiotherapy. One group of 13 patients received BCG and irradiated leukemic cells, and another 12 received this therapy plus CG once a week, given on the day of administration of BCG and tumor cells. Two-year survival was increased in the group receiving CG. Adults were given 1.5 mg of CG and children 0.75 mg of CG per injection.

Intravenous injections of CP have also been used clinically. Most studies have thus far dealt with the toxicologic and immunologic effects of CP (see below); however, several preliminary reports describing antitumor activity have already appeared. Band *et al.* (1975) treated 19 patients with various progressive metastatic solid tumors who had previously been treated with either chemotherapy, radiation therapy, or both. Doses of CP ranged from 0.5 to 6 mg/m^2 and were given by intravenous infusions daily for 10 days. Objective regressions were observed in 4 patients and include the following tumors: colon carcinomas, breast cancer, and pulmonary metastases of bone sarcoma. Israel *et al.* (1975) treated 20 terminally ill patients with various solid tumors in whom previous chemotherapy treatment had failed. Intravenous CP at a dose of 4 mg was given daily, 5 times a week, for 4–16 weeks. In 8 patients (40%) the tumors regressed to more than 50% of their original size. These were all metastases at various anatomical locations from lung adenocarcinoma, malignant melanomas, reticulum cell sarcoma, gastric cancer, testicular seminoma, and mediastinal teratoma. Partial regressions caused by CP were successfully maintained by subsequent chemotherapy and weekly subcutaneous injections of CP. Different immunological functions were evaluated during immunotherapy, and the most characteristic finding was a decrease in C3 complement which correlated directly with the clinical improvement of patients.

Impressive data on CP therapy of disseminated malignant melanoma were recently reported by Reed (1976). Single intravenous injections of CP at 5 mg/m^2 were given 19 days after chemotherapy with imidazole carboxamide (DTIC). In addition to CP, irradiated cultured melanoma cells (6×10^7 cells) were given subcutaneously. Seven of 11 patients (64%) treated in this way showed objective regression of liver metastases, compared with only 2 of 13 (15%) responses in patients treated with DTIC alone ($P < 0.05$). Patients who received the combined treatment lived longer than patients in the chemotherapy group: median survival 7.5 and 4 months, respectively. The effect on lung metastases was not as striking; 45% of patients responded to immunochemotherapy and 25% to chemotherapy.

Another study involving malignant melanoma showed that intravenous CP (2 injections of 5 mg/m^2 given a week apart) can increase the therapeutic effect of cyclophosphamide and DTIC (Presant *et al.*, 1976).

Single intravenous treatments with CP, 5 mg/m^2, decreased tumor growth and prolonged survival of patients with metastatic breast cancer (Minton *et al.*, 1976), and appreciably improved chemotherapy treatments of lung carcinoma (Takita and Moayeri, 1976; Valdivieso *et al.*, 1976).

Intratumor injection of CP, which is very efficient at producing complete regression of experimental animal tumors, has also been tried in patients (Cunningham-Rundles *et al.*, 1975; Hirshaut *et al.*, 1975; Israel, 1975). Israel (1975) reported that 11 out of 11 cutaneous melanomas and 1 out of 2 skin metastases of oat-cell carcinoma completely regressed when injected with 4–8 mg of CP for 5 consecutive days. Intratumor injections induced local inflammation within a few days, and this was sometimes followed by suppuration. Injections were also painful, causing discontinuation of prolonged treatment. In studies by Cunningham-Rundles *et al.* (1975), the mean maximum tolerated dose of intralesional CP was 18 mg. In their study 3 out of 14 injected tumors underwent complete regression and another 3 partially regressed. All six patients who responded to CP had tumors localized in the skin, whereas nonresponders had tumors which, in addition to the skin, involved subcutaneous tissues and visceral organs. The growth of uninjected tumors was not affected by this treatment.

B. Toxicity

Many Phase I clinical studies with CP have recently been conducted, and most of these have used the intravenous route of injection (Band *et al.*, 1975; Hirshaut *et al.*, 1975; Israel *et al.*, 1975; Ossorio *et al.*, 1975; Reed *et al.*, 1975b; Woodruff *et al.*, 1975; Cheng *et al.*, 1976; Fisher *et al.*, 1976b; Humphrey *et al.*, 1976; Minton *et al.*, 1976). Patients involved in these studies had various malignant tumors of very advanced clinical stages and were resistant to currently available therapies. The first study (Reed *et al.*, 1975b) employed the initial intravenous CP dose of 1 mg/m^2; after this dose was shown to be safe, 2, 3, 5, and 7.5 mg/m^2 were investigated. The bacteria were given by infusions in 5% dextrose in water over a period ranging from 1 to 4 hours. The doses of CP employed by other investigators were usually 5 mg/m^2 or lower and have been administered by infusion in 250–500

ml of either saline or 5% dextrose in water over 1–4 hours. We shall summarize the side effects of intravenous CP reported in the various published studies. For the initial 2–3 hours after the start of the infusion patients have no symptoms. A chill reaction then develops in most patients, which, in an appreciable number, can be vigorous, lasting from 20 to 40 minutes. Occasionally, recurrent chills may appear after a few minutes or hours after cessation of the first chill. A high proportion of patients develop peripheral vasoconstriction manifested as a mild cyanosis or blanched appearance. Following the onset of the chill reaction, mild hypertension may occur and an increase in the pulse rate is regularly observed. Toward the end of the chill period most patients develop a fever up to 105°F, and at this time most patients have nausea, vomiting, and mild headache. Some patients may feel slightly apprehensive. The temperature usually subsides within a few hours, but may last up to 3 days. Some patients sweat profusely during the regressive phase of the fever and some of them become mildly hypotensive. Myalgias and muscle weakness are rarer symptoms that develop at higher doses of CP. The overall symptoms associated with intravenous CP are usually more intensive with increasing doses of CP. Patients receiving a second intravenous dose of CP develop the same, but less intensive, symptoms. After a few repeated infusions of CP, the symptoms are minimal or may not appear at all. Even progressively increasing daily doses soon result in decreased toxicity symptoms. Fisher *et al.* (1976b) observed that a single intravenous administration of 100 mg of hydrocortisone 0.5 hour prior to CP infusion markedly diminished CP toxicity.

In most studies renal and hepatic functions were not affected. However, with very high single doses of CP (more than 20 mg per patient), slight rises in serum alanine aminotransferase levels and jaundice have been detected (Woodruff *et al.*, 1975).

A case of angina pectoris associated with a sudden rise of blood pressure (Band *et al.*, 1975) and of a generalized Schwartzman reaction with acute azotemia and severe thrombocytopenia (Reed *et al.*, 1975b) was also reported. Although cardiovascular changes have generally been minimal, they may be sufficient to contraindicate CP in patients with compromised cardiovascular status (Band *et al.*, 1975).

Most of the symptoms that accompany intravenous CP injection are also associated with subcutaneous injections, but in a milder form. Fever, headache, and malaise are observed most often. At the injection sites, soreness, induration, and erythema develop and may last from several hours to a few days (Reed *et al.*, 1975b). Local soreness has persisted occasionally for about a week. To counteract pain at the in-

jection sites, CP has been mixed with 2% xylocaine or 1% lignocaine (Israel and Halpern, 1972; Israel and Edelstein, 1975). Similar symptoms are associated with the intralesional injections of CP (Cunningham and Rundles *et al.*, 1975).

C. Immunologic Parameters

Various immunologic parameters have been monitored in patients undergoing therapy with CP. Total white blood cell counts are rarely affected; however, alterations in the absolute number of lymphocytes and monocytes frequently occur. A decrease is already apparent within a day of CP infusion, but cell numbers return to normal within a week (Ossorio *et al.*, 1975; Reed *et al.*, 1975a; Woodruff *et al.*, 1975; Cheng *et al.*, 1976; Minton *et al.*, 1976; Ochoa *et al.*, 1976). Several investigators have reported great variability in individual total lymphocyte and T-cell counts (Israel *et al.*, 1975; Hirshaut *et al.*, 1975; Humphrey *et al.*, 1976); however, Ochoa *et al.* (1976) found decreased lymphocyte and T-cell counts in all patients. The number of circulating B cells may be increased moderately (Hirshaut *et al.*, 1975).

The PHA responsiveness of peripheral blood lymphocytes is depressed 1–5 days after intravenous CP but then recovers, occasionally rising above the base line (Reed *et al.*, 1975a; Minton *et al.*, 1976). After 1–2 months, patients receiving multiple CP injections have shown increased reactivity to T-cell mitogens, PHA, and concanavalin A (Con A), whereas responses to poke weed mitogen and streptolysin "O" have remained unchanged (Reed *et al.*, 1975b). A similar tendency toward increased PHA and Con A reactivity has also been described by Israel *et al.* (1975).

The effects of CP on skin testing with delayed-hypersensitivity antigens: dermatophytin, candida, streptokinase-streptodornase, mumps, DNCB, and others have been monitored. In studies by Reed *et al.* (1975b), 9 of 25 patients receiving intravenous CP showed an improvement in skin test responses. Cheng *et al.* (1976) reported that 10 positive reactions were converted to negative and 4 negative reactions to positive. Variable results on skin tests were also reported by Israel *et al.* (1975) and by Hirshaut *et al.* (1975). Of 6 patients initially unresponsive to DNCB, 4 became positive to the same antigen shortly after receiving their first CP dose (Hirshaut *et al.*, 1975).

James *et al.* (1975) found a constant increase in the circulating IgG levels of all patients with malignant melanoma, breast or gastric cancer who had received 20 mg of CP intravenously followed by weekly intramuscular injections of 2 mg for 10–11 weeks. All 4 subclasses of

IgG were increased, but particularly IgG_2. IgA, IgM, and IgE levels were inconsistently affected. The rise in immunoglobulin levels was attributed largely to the development of antibodies against CP. Preexisting antibodies were noted in all patients and titers rose within 2 weeks after the initial CP injection and remained elevated throughout the 100-day period of observation. Development of anti CP antibodies in CP-treated patients has also been observed by Minton *et al.* (1976), and they found no correlation with the antitumor activity of CP. Other studies dealing with the effects of CP on antibody production in patients with various malignant tumors have shown no significant changes in immunoglobulin levels (Dimitrov *et al.*, 1975b; Israel *et al.*, 1975; Hirshaut *et al.*, 1975). Only occasionally IgE (Woodruff *et al.*, 1975) and IgM (Hirshaut *et al.*, 1975) may be elevated.

Intravenous CP may be associated with complement activation in cancer patients. Serum levels of C3 (Dimitrov *et al.*, 1975b; Israel *et al.*, 1975) and C3 and C4 (Biran *et al.*, 1975) have been found to be decreased after intravenous CP. A similar depletion in C3 and C4 levels has occurred in normal human serum *in vitro* after addition of CP. Addition of CP to guinea pig serum, which, unlike the human serum did not contain antibodies against CP, only resulted in decreased C4 levels (McBride *et al.*, 1975c). The depletion of C3 and C4 levels in patients occurred more frequently if the patients were immunocompetent, and the suggestion is that CP activates complement in patients both directly via the alternative pathway (C3), and via the classical pathway (C1, 4, 2, . . .) when natural or induced anti-CP antibodies are present (Biran *et al.*, 1975; McBride *et al.*, 1975c). Israel *et al.* (1975) found that decrease in C3 levels in the serum of patients correlated directly with the effectiveness of CP therapy. We have already discussed a possible role for C3 cleavage products in the activation of mouse macrophages (Schorlemmer *et al.*, 1976).

IX. Perspectives and Prospects

The results achieved using CP in the treatment of experimental animal tumors have been impressive, and the overall clinical results to date are encouraging. Other than for the toxicity studies, however, the human data are as yet too preliminary to form the basis for discussing future perspectives. For this we have drawn on the large amount of animal data available, but, in doing so, are aware that there are, thus far, no human data indicating whether or not what we consider to be the modes of action of CP in animals, also operate in man. Results concerning the *in vitro* performance of macrophages and lymphocytes

from CP-treated patients in both nonspecific and specific antitumor assays are eagerly awaited.

Tumor size is a critical consideration in CP immunotherapy. Throughout the animal studies, small tumor masses, even strongly antigenic ones, have responded best to treatment. Clinically these situations are likely to be seen in patients with newly diagnosed tumors at early stages, or those whose tumor burden has been reduced by prior irradiation, surgery, or chemotherapy.

The combination of CP with chemotherapy in the treatment of some animal tumors has been extremely successful, the individual antitumor effects being additive, or even synergistic. Clinical studies using CP as an adjunct to chemotherapy have been preliminary, but at least they indicate that immunostimulant therapy is not incompatible with immunosuppressive chemotherapy. The combination chemoimmunotherapy approach is likely to benefit from further animal studies using appropriate tumor models. Screening for potential synergistic or antagonistic CP-drug combinations could be rewarding. It may be that CP potentiates the antitumor activity of some drugs as it does the effects of local irradiation, or that CP-mediated antitumor effects, once established, are sensitive to subsequent drug treatment.

It is apparent that some of the therapeutic effects of CP may not be a direct result of its antitumor activity. Infection is the cause of death in some cancer patients immunosuppressed either directly as a result of the disease, or because of conventional therapies. Increased survival of any such patients receiving CP may be attributable, at least in part, to enhanced bacterial resistance resulting from reticuloendothelial stimulation. Another "indirect" antitumor effect might be that more intensive radio- or chemotherapy can be tolerated because of CP-mediated protection against their myelodepressive side effects.

Small disseminated microfoci of tumor cells persisting or appearing after successful treatment of the primary tumor mass are often the ultimate cause of death in cancer patients. It is for this reason that the establishment of systemic antitumor immunity should be the ultimate goal of any immunotherapeutic regimen. The designers of clinical trials involving CP should take into consideration the fact that different kinds of systemic antitumor immunity (specific or nonspecific) may be preferentially selected under different circumstances. The intensity and durability of the specific cell-mediated antitumor immunity that has resulted from local interaction of CP and tumor antigen in mice suggests this to be a desirable situation to achieve clinically. Optimal immunity may be restricted to situations where either direct intralesional injection or stimulation of the tumor-draining nodes is practical,

or specific active immune therapy using CP mixed with attenuated tumor cells when these are obtainable. In some cases a surgically resectable tumor may be accessible to preoperative intralesional CP injection, e.g., gastric cancers via endoscopy, thereby establishing specific tumor immunity that may not result from surgical resection alone. A reasonably strong tumor-associated antigen and high degree of patient immunocompetence would both be expected to facilitate specific immunization. Animal studies indicate that optimal immunization requires a balance between the amount of CP and tumor antigen. However, given our poor understanding of the antigenicity of human tumors, clinical trials attempting specific immunization must be empirical.

The immunologically nonspecific, macrophage-mediated, systemic antitumor activity that seems to predominate after systemic injections of CP does not require close contact between CP and tumor and is likely to be independent of tumor antigenicity and patient immunocompetence. Theoretically, therefore, in the case of tumors at inaccessible sites, those of low antigenicity, and patients with poor immunocompetence, nonspecific therapy may be appropriate. Anergic patients may benefit particularly from the antibacterial effects that have been described for systemic CP. Although little nonspecific systemic stimulation has resulted from a single subcutaneous injection of CP in mice, the systemic effects of repeated subcutaneous injections have been shown to be additive. The final degree of stimulation achieved, however, was less than for the same amount of CP given as repeated systemic injections (Scott and Warner, 1976). In widely disseminated tumors, the wider distribution of CP following systemic injection would be expected to maximize the chance of CP reaching either tumor sites or draining nodes, thereby engaging specific immune mechanisms as well. Combination of systemic and regional CP injections designed to elicit both nonspecific and specific immunity may prove to be particularly effective. Suit *et al.* (1976b) found combination of intralesional and systemic CP to be more effective in mice than either individual treatment. Scott (1974c), however, has shown that systemic CP may diminish the effectiveness of subsequent intralesional therapy, although it did not diminish the ability of CP-irradiated tumor cell mixtures to immunize (Scott, 1975b).

The individual doses of CP that can be administered systemically in humans are considerably less than those required for effective tumor therapy in mice. However, unlike the mouse situation with rapidly growing experimental tumors, the human situation allows for repeated injections over an extended period. The effects of repeated low doses,

both systemic and subcutaneous are additive in the mouse and interestingly the antitumor protection afforded by repeated low doses of CP may be greater than the total dose given as a single injection (Milas *et al.*, 1975c). In specific cell-mediated immunity resulting from CP-irradiated tumor cell mixtures in mice, the doses of CP have been in the human equivalent range (Bomford, 1975; Scott, 1975b).

Despite the fact that experimental conditions in CP animal tumor studies have been extremely uniform—i.e., the same tumor cells, in similar sites in genetically uniform, often specific pathogen-free animals—the responses of individual tumors to CP have varied. Some have regressed completely and some partially, whereas others have been unaffected (Milas *et al.*, 1974d). That CP responsiveness is affected also by genetic constitution is apparent from the differing degrees of lymphoreticular stimulation that occur in different strains of inbred mice (Stiffel *et al.*, 1970). This may be associated with differing degrees of natural immunity to CP. All these considerations would indicate that, in the considerably less uniform human situation, a wide range in the responses of "similar" tumors to CP therapy may be expected.

The identification of clinical situations in which CP may be effective is a major problem. It is apparent, however, that the basic mechanisms underlying the antitumor activity of CP, i.e., macrophage activation and potentiation of specific cell-mediated immunity, are similar to those described for BCG (see review by Bast *et al.*, 1974). The clinical assessment of BCG is more advanced than that of CP, and, given the basic similarities between the two organisms, the evaluation of CP in situations where results with BCG have been promising seems logical.

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