# IN VITRO STUDIES ON CORYNEBACTERIUM PARVUM-INDUCED ACTIVATION OF ANTI-TUMOUR POTENTIAL IN MURINE MACROPHAGES

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To my wife

PATRICIA

who helped in so many ways

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## ABSTRACT

Killed vaccines of the adjuvant *Corynebacterium parvum* (*Propionibacterium acnes*) have proved to be potent anti-tumour agents in a number of experimental systems and are currently being assessed clinically, either alone or as an adjunct to other cancer treatments. In animals, systemically administered *C. parvum* can exert a strong, non-specific anti-tumour action which is thought to be mediated principally by activated macrophages. The author describes *in vitro* experiments analysing the possible pathways leading to the production of such activated macrophages in *C. parvum*-treated CBA mice. The tumour studied was a methylcholanthrene-induced fibrosarcoma.

It was found that spleen cells from mice treated intravenously, or intraperitoneally (but not subcutaneously) with *C. parvum* could induce, *in vitro*, anti-tumour cytotoxicity in normal, non-cytotoxic, murine peritoneal macrophages, provided that *C. parvum* was also present. This requirement for the presence of *C. parvum* was shown to be a specific one, other strains of *C. parvum* or other unrelated bacteria being ineffective. The macrophage-activating cell was found to be radio-sensitive, but resistant to treatment with sodium aurothiomalate or anti-macrophage serum. However, exposure of normal peritoneal macrophages to sodium aurothiomalate, either before, or during activation with spleen cells, abrogated the cytotoxic effects.

Nylon wool column separation of spleen cells into B cell-rich and B cell-depleted fractions indicated a possible requirement for B cells in the activation process. On the other hand a T cell requirement was shown by the failure of spleen cells from *C. parvum*-treated T cell-deprived mice to activate macrophages *in vitro*. Treatment of spleen cells with anti-θ serum prior to the activation process yielded inconclusive

evidence with regard to the T cell dependence of macrophage activation.

Spleen cells capable of activating macrophages *in vitro* did not appear in the *C. parvum*-treated mouse until after day 6 post *C. parvum* injection. This contrasted with the early appearance, by day 3, of cytotoxic spleen and peritoneal cells in i.p.-treated mice. This suggested the existence *in vivo* of two separate pathways leading to the stimulation of anti-tumour effector cells.

*In vitro* induction of anti-tumour activity could also be achieved using locally-stimulated lymph node cells, or cell-free supernatants from mixed cultures of *C. parvum*-sensitised spleen cells and *C. parvum*.

During the course of *in vitro* studies on *C. parvum*-activated macrophages it was observed that such macrophages lost their cytotoxic capacity on culture. This loss of activity could be delayed by the addition of *C. parvum* to the cultures. This maintenance effect by *C. parvum* was found to be radioresistant, sensitive to sodium aurothiomalate, T cell dependent, and at least partially specific.

The significance of these results and the possible mechanisms underlying them have been discussed in relation to the existing knowledge of *C. parvum*'s biological and anti-tumour effects.

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# I N T R O D U C T I O N

## 1. HOST RESISTANCE TO TUMOURS

A central objective of research into the therapy of cancer is the elucidation of those features of tumours which distinguish them from normal tissue and which enable them to escape the body's usual homeostatic tissue restraints. Such differences between malignant cells and normal cells can then provide a basis for diagnostic and therapeutic purposes, and, even for prophylaxis. However, it is only in comparatively recent times that this concept of cancer has attained universal acceptance since, apart from being supplied by the body of nutrients and oxygen, tumours had often been regarded as being autonomous, growing without regard to the well-being of the organism as a whole. Nevertheless, it was well known that tumours did not always progress inexorably and could in fact be held in check or even repulsed by unknown homeostatic mechanisms. Much of this evidence is anecdotal and concerns regression or progression of tumours in individual cases. From an extensive review of cancer literature between 1900 and 1965, Everson and Cole (1966) cite 176 cases sufficiently well documented to provide convincing proof of spontaneous regression of established, untreated tumours. Some of the regressions were permanent, others merely transient. In cases of melanoma, for example, certain tumour nodules are frequently seen to be regressing while others on the same limb are developing (Bodenham, 1968). Many tumours such as breast carcinoma and melanoma may suddenly reappear after many years of inactivity following initial diagnosis and treatment of the primary lesion (Willis, 1973). Occasional reports have appeared of cases in which tumour metastases have regressed following surgical removal of the primary tumour

(Rosenberg, Fox and Churchill, 1972) or following a febrile illness or infection, particularly infections involving the tumour site or its draining node (Nauts, Swift and Coley, 1946; Everson and Cole, 1966).

Direct evidence of one aspect of homeostatic tumour control came with the clinical and experimental observations of Huggins illustrating the hormone dependence of certain tumours of prostate, breast, thyroid and kidney (Huggins and Hodges, 1941; Huggins, 1967). In addition to hormonal control tumour cell growth may be governed locally by tissue specific chalones (Bullough, 1975; Forscher and Monck, 1973). Mere physical restraints may also play an important part in tumour containment, tumour nodule size and subsequent metastatic spread depending on angiogenesis (reviewed by Folkman, 1974) and tumour invasiveness.

The idea that immune mechanisms might play a part in the resistance to and rejection of neoplasia developed as a consequence of the awakening interest in immunological resistance to microbial pathogens during the latter decades of last century. Central to this idea was the expectation that tumour cells would be antigenically different from host tissue and, as with bacterial infection, immunotherapeutic procedures could be developed against these foreign antigens. The search for an effective immunotherapy of cancer continues to this day. One such approach has involved the administration of agents which potentiate specific and/or non-specific immunity. The possible mechanisms involved in the anti-tumour effects of one such agent *Corynebacterium parvum* provides the subject for this thesis. The evidence for an active participation by the immune system in

the host's defence to tumours, and the prospects for successful immunological intervention in cancer, particularly with agents such as C. parvum, are outlined in the remainder of this introductory review.

## 2. TUMOUR ANTIGENS

Unaware of the principles of allograft rejection and of histo-compatibility, researchers in the first quarter of this century believed they were studying tumour-specific immunological responses when they transplanted tumours. The unequivocal demonstration that tumours could be antigenically different from their original host had to await the development of in-bred strains of laboratory animals during the 1940's.

### 2.1 Tumours induced by chemical carcinogens

Tumour associated transplantation antigens (TATA) were first demonstrated on methylcholanthrene-induced sarcomas in mice (Gross, 1943; Foley, 1953; Prehn and Main, 1957). Ligation or excision of tumour transplants in syngeneic mice resulted in resistance to subsequent challenge by the same tumour. That the induced resistance was not due to histo-compatibility differences between tumour and host was proved by experiments in mice bearing primary tumours (Klein, Sjögren, Klein, and Hellström, 1960).

The TATA of chemically-induced neoplasms are generally unique, in sharp contrast to those of virally-induced tumours. For example, tumours induced independently in the same host do not cross react

antigenically (Globerson and Feldman, 1964). However, evidence for shared TATA among chemical induced tumours has been reported (Reiner and Southam, 1967; Taranger et al., 1972). Subsequent infection of tumours with an oncogenic virus would also lead to expression of virus associated tumour antigens (Stück, Old and Boyse, 1964; Klein, 1969). Foetal antigens may also be expressed on chemical-induced tumours (Baldwin, Glaves and Vose, 1972; Baldwin, Embleton, Price and Vose, 1974).

## 2.2 Tumours induced by viruses

Tumours induced by the same virus share common TATA (Habel, 1961; Sjögren, Hellström and Klein, 1961; Klein and Klein, 1964). DNA virus-induced TATA are distinct from the actual viral antigens (Law, 1970). Differentiation between RNA virus antigens and tumour antigens is difficult to establish since most of the tumour cells produce infectious virions which bud from the cell surface (Law, 1970). However, there is evidence that there are virally determined cell surface antigens distinct from the viral envelope antigens (Jonsson and Sjögren, 1965; Levy et al., 1969; Lilly and Steeves, 1974). In addition to the common virus-associated antigen, tumours such as the virus-induced mammary tumour may have other distinct TATA (Vaage, 1968; Morton et al., 1969).

## 2.3 Differentiation antigens

Differentiation antigens represent the re-expression in a tumour cell of certain genes which are normally only operative at a certain

stage in the differentiation of a cell from its embryonic stage to specialised cell. De-repression of genes may be a fundamental process in malignant transformation. Two such differentiation antigens are the TL and Gix antigens normally expressed on thymocytes from certain mouse strains, but absent from others. TL-negative mice with leukaemia often express the TL antigen on their leukaemic cells (Boyse, Old and Stockert, 1968). Similarly, the Gix antigen can be induced in Gix-negative rat or mouse thymocytes by infection with Gross murine leukaemia virus (MuLV) (Stockert, Old and Boyse, 1971; Stockert et al., 1972). Both these systems are thought to represent de-repression in negative strains of mice of genes coding for TL or Gix. Many tumours express other normal antigens and some do so in greater quantity than normal tissue (Fogel and Sachs, 1962).

## 2.4 Embryonic (EA) or Foetal (FA) antigens

Embryonic or foetal antigens are expressed on many tumours including virus-induced (Coggin et al., 1970; Ting et al., 1972) and chemical-induced tumours (Baldwin et al., 1974). Certain embryonic antigens may be common to several different tumours (Thomson and Alexander, 1973; Ting et al., 1972). Immunisation with foetal tissue evokes immunity to subsequent tumour challenge in some systems (Coggin et al., 1970; Girardi et al., 1973; Grant et al., 1974) but not in others (Ting et al., 1972; Baldwin et al., 1974). This discrepancy may be due to the failure to select embryo cells during the limited period in which these particular antigens appear in

embryogenesis (Coggin *et al*., 1970; Baldwin *et al*., 1972). Brawn (1970) demonstrated that lymph node cells from multiparous mice were cytotoxic *in vitro* to several methyl-cholanthrene-induced sarcomas.

## 2.5 Antigens on human tumours

A distinctive feature of many human tumours is that they carry cross-reacting organ or tissue-specific antigens. Thus lymphocytes from individuals with a particular cancer can by cytotoxic or growth inhibitory *in vitro* to tumour cells from another individual provided it is of the same histologic type (Hellström, Hellström, Sjögren and Warner, 1971; Hellström, Warner, Hellström and Sjögren, 1973). These antigens may be differentiation or embryonic antigens.

Two tumour-associated macromolecules have received a great deal of attention in recent years, carcinoembryonic antigen (CEA) and alpha-foetoprotein (AFP). These antigens can be detected in the serum of cancer patients and, although of doubtful immunogenicity, would appear to be of value as cancer diagnostic aids (see Zamchek and Kupchik, 1976; McIntire and Waldmann,1976).

## 2.6 Immunogenicity

It is recognised that the so-called spontaneous tumours, tumours of unknown aetiology, both in man and animals, are generally non-immunogenic (Main and Prehn, 1957; Baldwin, 1966; Peters, 1975). Immunogenicity is usually assessed by tumour transplantation into

syngeneic animals previously exposed to that tumour. Only immunogenic tumours will immunise against such subsequent challenge. Chemical-induced tumours can also be non-immunogenic (Baldwin, 1973; Baldwin and Embleton, 1969). Prehn (1975) demonstrated that both immunogenicity and the latent period between exposure of tissue to the carcinogen and appearance of the tumour, were dependent on the dose of the carcinogen: as the concentration was decreased the latent period lengthened and immunogenicity declined.

The fact that so much experimental work in tumour biology is conducted using highly immunogenic tumours has resulted in considerable concern over the desirability of studying poorly immunogenic tumours which would more closely reflect those neoplasms encountered clinically (Klein and Klein, 1977; Hewitt, Blake and Walder, 1976; Weiss, 1977).

## 3. TUMOUR IMMUNOLOGY

If so many tumours do possess neoantigens or TATA, and hence are potential targets for the immune system, it would be expected that immune mechanisms would play an important part in the host's resistance to that neoplasm. Indeed, there is now a vast body of evidence on the interaction of the immune system and malignant tumours. Some of this is circumstantial, such as the clinical observations of regression mentioned earlier, and which is indicative of host resistance but gives no insight into the mechanisms involved. Other circumstantial evidence has come from histological examinations of tumour deposits and lymph nodes in cancer patients and tumour-bearing animals.

3.1 Histological evidence

Many malignant tumours are infiltrated by lymphoreticular cells: lymphocytes, macrophages, polymorphonuclear leucocytes, plasma cells, mast cells. This stromal response to neoplasia has been of interest to pathologists since the latter half of the 19th century (see Underwood 1974 for review) and many believed the phenomenon was indicative of host defence against the tumour (Da Fano, 1912; Murphy, 1926). Attempts to correlate lymphoreticular infiltration with favourable prognosis have been successful with certain tumours such as carcinoma of the breast (Hamlin, 1968) and oesophagus (Takahashi, 1961) seminoma (Dixon and Moore, 1953) and many more (Underwood, 1974). Both positive (Thompson, 1972) and negative (Little, 1972) correlations have been reported for malignant melanoma. Sinus hystiocytosis of draining lymph nodes of tumours has also been reported (Black, Opler and Speer, 1954) but the diagnostic or prognostic value of this observation is now in doubt (see Silverberg, Frable and Brooks, 1973). A recent study using immunofluorescent techniques showed that T cells predominate in the infiltrates of primary human tumours but that B cells predominate in certain metastases (Husby, Hoagland, Strickland and Williams, 1976). However, many studies of human and animal tumours have also demonstrated the macrophage to be an important constituent (up to 50%) of tumour infiltrates (Evans, 1972; Gauci and Alexander, 1975; Szymaniec and James, 1976; K. Moore and Moore, 1977). The magnitude of the cellular infiltrate correlates well with tumour immunogenicity (Evans, 1972; K. Moore and Moore, 1977). The significance of the high macrophage content of some tumours is discussed more fully below.

3.2 Immune surveillance

The last two decades has seen intensive research into the role of the immune system in tumour biology. The most important concept, and indeed the central dogma of tumour immunology in this period, has been that of immune surveillance. This concept originally phrased by Ehrlich (1909) and later developed by Thomas (1959), Burnet (1964, 1967, 1970a, b, 1971) and others (Good and Finstad, 1969), was that tumours arise at a much more frequent rate than clinical evidence would allow but that the majority of these tumours are recognised as foreign and eliminated soon after initiation by the immune system. Failure of this surveillance mechanism results in malignant tumour growth. Thomas (1959) actually proposed that the raison d'être of cell-mediated immunity, as observed in allograft rejection, was in fact a primary defence mechanism against neoplasia.

An important consequence of the theory, and one which ought to provide much of its supporting evidence, is that individuals with congenitally defective immunological responses or individuals undergoing immunosuppressive therapy (e.g. transplant recipients) should exhibit a greatly increased incidence of malignant tumours (Good and Finstad, 1969; Keast, 1970; Gatti and Good, 1971; Waldmann et al., 1972). Alternatively, the development of a malignant tumour in an individual should have been associated with, or presaged by, some immunological deficiency. It should be noted that initial failure of immunosurveillance does not imply that an immune response is not subsequently evoked by the tumour. Evidence dealing with the immunology of established tumours will be dealt with in a later section.

### 3.3 Immunodeficiency - Clinical Evidence

There have been several analyses of the proposed link between immunodeficiency and cancer (Good and Finstad, 1969; Gatti and Good, 1971; Kersey, Spector and Good, 1973a, b; Melief and Schwartz, 1975). Patients with the Wiskott-Aldrich syndrome or ataxia-telangiectasia exhibit an increased incidence of lymphoreticular tumours while patients with Bruton's agammaglolbulinaemia have a greater chance of developing leukaemia (Kersey et al., 1973b). A few individuals with severe combined immunodeficiency (Kersey et al., 1973b) and also those with the Chediak-Higashi syndrome (Windhorst, Zelickson, and Good, 1966) have been reported to develop lymphoreticular malignancy. Collation of cases at the Immunodeficiency - Cancer register in Minnesota, USA showed that 58% of patients had lymphoreticular tumours, 17% had leukaemias and 18% had epithelial tumours (Kersey et al., 1973a).

A higher incidence of malignancy has also been reported in connection with certain genetic disorders such as Fanconi's anaemia (Swift and Hirschhorn, 1966) and Klinefelter's syndrome (Fraumeni and Miller, 1967), diseases in which no detectable immune deficit has been detected. This observation may be accounted for by the extraordinary susceptibility of fibroblasts from these patients to *in vitro* viral transformation (Todaro, Green and Swift, 1966; Mukerjee, Bowen and Anderson, 1970).

Individuals on immunosuppressive therapy also exhibit an increased frequency of malignancy. Hoover and Fraumeni (1973), analysing data from 6,297 renal-transplant recipients, observed that the risk of developing one of the lymphomas was 35 times

higher than normal and that with the most prominent type, reticulum-cell sarcoma, the risk was increased 350-fold. Skin and lip cancers also had a statistically greater incidence in these individuals. An unusual feature of the lymphomas and which has not been adequately explained (see Stutman, 1975a; Melief and Schwartz, 1975), was their extraordinary frequency in central nervous tissue.

The predominance of lymphoreticular malignancies in the above studies detracts from the support they are alleged to give to the immune surveillance concept; a greater incidence of all tumour types would be expected in the absence of a healthy surveillance system. Several explanations for the frequency of lymphoreticular malignancy have been proposed. Schwartz (1975) has pointed out that these tumours are normally very rare (Silverberg and Grant, 1970) and that their relatively high immunogenicity would ensure early elimination by the immune system, unless it was depressed. It has also been argued that chronic antigenic stimulation from infection (or the graft itself in transplant recipients) may lead to development of lymphoreticular malignancy (Ten Bensel, Stadlam and Krivit, 1966; Krueger, 1972). This would arise because of the lack of immunoregulatory feedback mechanisms (eg. T suppressor cells) and/or activation of latent oncogenic viruses within activated lymphocytes (Schwartz, 1972, 1975). In this respect it is interesting that Lukes and Collins (1974) regard the reticulum-cell sarcoma as consisting of cells morphologically similar to antigen-stimulated lymphocytes and not reticulum cells.

It should be noted that in patients with other disease states, such as rheumatoid arthritis and myasthenia gravis, and who are

being treated with immunosuppressive drugs, the incidence of new tumours is much less dramatic than in organ transplant recipients, and in many studies no positive correlation has been found (Brendel, Seifert, Lob et al., 1972; Pirofsky et al., 1972; see also Stutman, 1975a).

Association between cancer incidence and those periods of life during which immune responses may be poorly developed or defective such as in old age or the perinatal period, has often been quoted in support of the immune surveillance theory (Gatti and Good, 1971; Keast, 1970). Doll and Kinlen (1970) agree that in general there is a peak incidence of malignancy in infancy or childhood and that following adolescence there is a fairly regular increase to old age. However, they also quote many discrepancies; seminoma, Hodgkin's disease, carcinoma of the cervix. In addition, it must be remembered that many aged individuals are entirely immunocompetent (Hallgren et al., 1973), and that immunological responses have been detected in the human foetus (Silverstein and Lukes, 1962). Doll and Kinlen (1970) went on to point out that tumour incidence correlated more closely with length of exposure to carcinogens as shown by leukaemia incidence in patients exposed to irradiation for ankylosing spondylitis (Doll and Kinlen, 1970), cancer of lung in asbestos workers (Knox, Holmes, Doll et al., 1968) and cancer of bladder in workers exposed to naphylamine or benzidine (Case et al., 1954).

### 3.4 Development of tumours in thymectomised or ALS-treated animals

The literature contains many studies on the effects of neonatal

thymectomy and anti-lymphocyte serum (ALS) on tumour development, spontaneous as well as viral- and chemical-induced tumours. An extensive review by Stutman (1975a) illustrated that many of these reports provide conflicting results with respect to the role of immunity in host defence of neoplasia. Many of the discrepancies between experiments may be accounted for by differences in carcinogen used, dose, strain of animal and on the immunosuppressive effects of the carcinogen.

### 3.4.1 Chemical-induced tumours

Mice thymectomised in early life and injected as young adults with methylcholanthrene (MC) subcutaneously generally showed no increased incidence of local tumours (Stutman, 1975a). In contrast, thymectomy of mice together with exposure to chemicals such as urethane resulted in an increase in lung adenoma formation (Ribacchi and Giraldo, 1966; Trainin and Linker-Israeli, 1969, 1970). Grafting of thymus back into these mice normalised the incidence of lung adenoma (Trainin and Linker-Israeli, 1970). Results with ALS follow much the same pattern as for thymectomy with data appearing for and against immune surveillance (see Stutman, 1975a). One possible explanation for the lack of effect in some experiments using these procedures might be that the carcinogens are immunosuppressive anyway and no additional effects of immune deprivation would be observed (Kripke and Borsos, 1974). However, Stutman (1973) was able to distinguish between carcinogenic effects and immunosuppressive effects by varying the dose of carcinogen used.

3.4.2 Virus-induced tumours

The thymus dependence of host resistance to many oncogenic viruses has been demonstrated repeatedly. Neonatal thymectomy enhances the tumour incidence resulting from oncogenic viruses in hamsters (Yohn, Funk and Grace, 1968; Van Hoosier, Gyst and Trentin, 1968), rats (Allison and Taylor, 1967; Vandeputte and De Somer, 1965), and mice (Miller, Ting and Law, 1964; Law, 1966a, b; Allison, 1970). Furthermore, experiments in which post-thymectomy immune deficiency was restored confirmed that the immune system plays a major part in the host resistance to polyoma virus oncogenesis (Law, 1966a, b; 1969; 1970).

Immunosuppression by ALS treatment has also enhanced tumour induction in many experimental systems: in mice by adenovirus (Allison, Berman and Levery, 1967); and polyma virus (Allison and Law, 1968); in rats by polyma virus (Vandeputte, 1968); and in hamsters by SV40 (Tevethia, Dreesman, Lausch and Rapp, 1968). However, there are also ALS results discordant with these (see Stutman, 1975a). Explanations for the discrepancies can probably be found in the many variables affecting results of viral oncogenesis and additionally there is the problem that immunosuppression can lead to elevated, unchanged or decreased levels of interferon (Glasgow, 1971). Experimental systems involving leukaemias and lymphomas are also complicated by the possible effects of ALS on the tumour target cells themselves.

Despite these problems it can be said that, in general, neonatal tymectomy or ALS treatment facilitates viral oncogenesis and thus these results provide strong support for the immune surveillance

theory. Klein (1973, 1975) has argued that the immune system's competence to resist the effects of oncogenic viruses compared to its relative inability to deal with chemical-induced tumours could be explained by the relatively recent (in evolutionary terms) appearance in the environment of such chemical carcinogens. Immune protective mechanisms against tumour viruses may have evolved over a long period of time and may be generally transmitted by immune responsiveness genes (Klein, 1975).

An important distinction in studies of viral oncogenesis is whether the observed thymus dependence of host resistance is directed at the virus itself, at tumour antigens, or both. Dissociation of anti-viral and anti-tumour immunity has been discerned in Marek's disease of fowl (Powell and Rowell, 1977) and in an oncogenic herpes virus system (Laufs and Steinke, 1976).

### 3.4.3 Spontaneous tumours

The influence of neonatal thymectomy on the development of spontaneous tumours has also produced conflicting reports. Negative correlations were found by Sanford _et al_. (1973) and Burstein and Law (1971), whereas Cornelius (1971) found an increased incidence of lymphoreticular sarcomas in thymectomised mice. Thymectomy has been found to actually decrease the incidence and prolong the latent period of virus-induced 'spontaneous' mammary tumours (Martinez, 1964; Law, 1969). The reasons for this phenomenon are not clear but may involve hormonal or immunostimulation (_vide infra_) dependencies.

### 3.5 Development of tumours in nude mice

Although they can respond to some antigens and possess some theta-positive cells, homozygous (nu/nu) nude mice, nevertheless, have a greatly impaired immune system making them highly susceptible to infection and incapable of rejecting allografts, and even xenografts (Wortis, 1974). The risk of developing spontaneous tumours should, therefore, be high in these animals following predictions from the immune surveillance theory. However, observation of thousands of nude mice over their life span (7 months in conventional housing and approaching that of normal mice in germfree or pathogen free environments) only found an increased incidence of lymphoreticular tumours (Outzen *et al*., 1975; Rygaard and Povlsen, 1976). Reasons why this tumour type is not acceptable evidence for the operation of immune surveillance have been discussed in an earlier section.

There seems to be no increase in tumours induced in these mice by polycyclic hydrocarbon carcinogens (Outzen *et al*., 1975; Stutman, 1974), and, with the exception of polyma virus-induced tumours, susceptibility to viral oncogens is no greater than in control (nu/+) mice (Stutman, 1975a). It must be recognised that nude mice may have alternative defence mechanisms mediated by B cells and/or macrophages. Stutman (1975b), for example, demonstrated that age-dependent resistance to polyoma virus in the nude mouse was mediated by non-T cells.

### 3.6 Immunologically privileged sites

Various immunologically privileged sites are known in experimental animals where first set allografts or xenografts are permitted. These

sites are the brain, hamster cheek pouch, anterior chamber of the eye, and the mouse mammary fat pad. Tumours might be expected to arise more frequently or be induced more readily in these sites in the absence of immunological surveillance. However, this expectation is not fulfilled by the available evidence. Spontaneous tumours of the brain are rare in laboratory mammals (Luginbuhl, Frankhauser and McGrath, 1968) and induction of tumours in both brain and hamster cheek pouch is often more difficult than in other tissues. In addition, any resulting tumours develop either more slowly than, or at a comparable rate to equivalent tumours in non-immunologically priveleged sites (Stutman, 1975a).

### 3.7 Host-tumour immunological interactions

The existence of tumour associated antigens and of immune responses to them, at least in experimental tumour systems, together with the circumstantial clinical evidence provide a powerful case for the involvement of immunological processes in the host's reaction to malignant tumours. The evidence for an immunological surveillance function as envisaged by, say, Burnet (1970a, b; 1971) is not, however, entirely convincing but does not detract from the demonstration of immune responses to established tumours. It is probable that a modified immunosurveillance theory will emerge encompassing non-specific anti-tumour effects mediated by macrophages and null cells (*vide infra*) in addition to specific T cell effects.

There is now a vast literature on tumour immunology. The following sections briefly outline this knowledge and the ways in which it has been obtained. Several techniques are available for the demon-

stration of humoral and cell-mediated responses to tumours.

## 4. HUMORAL IMMUNITY

### 4.1 Immunofluorescent and antiglobulin binding assays

Anti-tumour antibodies can be detected by immunofluorescent techniques (Möller, 1961) either directly (Goldstein, Klein, Pearson and Clifford, 1969) or indirectly (Klein, Clifford, Klein and Stjernswärd, 1966). For example, antibodies directed at melanoma cells have been detected in the sera of melanoma patients (Morton, Malmgren, Holmes and Ketchum, 1968). An alternative to this assay is the radiolabelled anti-globulin binding test (Burdick, Cohen and Wells, 1973; James *et al*., 1976, 1977). The presence of antibodies on tumour cells can lead to complement-dependent or cellular-dependent growth inhibition or lysis of tumour cells as demonstrated *in vitro* (*vide infra*). The significance of anti-tumour immunoglobulins *in vivo* is still in some doubt. Blocking and enhancing antibodies and antigen-antibody complexes will be discussed in a later section.

### 4.2 Complement fixation and complement-dependent cytotoxic antibody

Both complement-fixation (Eilber and Morton, 1970; Morton, Eilber, Malmgren and Wood, 1970) and complement-dependent cytotoxic antibody (Bloom, 1970; Fritze, Kern and Pilch, 1974) tests have been used to assess anti-tumour antibodies in patients' sera. Complement-fixing antibody levels may correlate with the clinical course of

neoplastic disease as, for example, in the case of melanoma (Morton et al., 1970), but in general, the significance of such assays to in vivo events is open to criticism particularly since positive results usually require use of xenogeneic serum for the complement source.

Antibody-dependent cell-mediated (K cell) cytotoxicity is dealt with under effector cells.

4.3 Inhibition of tumour mobility and re-aggregation

The presence of anti-tumour antibodies can also be detected by their effect on in vitro tumour cell behaviour. Currie and Sime (1973) showed that the in vitro movement of lymphoma cells could be inhibited by serum from syngeneic mice immune to those lymphoma cells. Currie (1976) also described a system in which tumour cell aggregation in vitro is inhibited by the presence of tumour-immune sera.

4.4 Lymphokines

A number of pharmacologically active, non-antibody substances, known collectively as lymphokines (Dumonde et al., 1969) are produced and released by immune lymphoid cells responding to specific antigen. Many of these lymphokines, such as lymphocyte mitogenic factor, lymphotoxin, skin reactive factor, chemotactic factor, macrophage migration inhibitory factor, and macrophage activation factor, mediate and regulate cellular immune responses including those to tumour cells. (For a review on lymphokines see Dumonde et al., 1975). Some of the above lymphokine effects are possibly mediated by the same

molecular moiety, although in most cases they can only be classified by biological function. For example, there is some evidence that macrophage migration inhibitory factor and macrophage activation factor are the same entity (Nathan, Remold and David, 1973). Lymphokine preparations have been used successfully as intradermal injections to treat cutaneous metastases in patients with adenocarcinoma of the breast (Papermaster et al., 1974; 1976).

Another group of lymphocyte-derived mediators called transfer factors can confer specific cell-mediated responses to a number of antigens, including tumour cells, when injected into non-immune recipients. Transfer of such responses has been demonstrated by delayed hypersensitivity skin tests and various in vitro assays such as lymphocyte transformation and macrophage migration inhibition (Lawrence, 1969). A number of studies using transfer factor preparations in cancer therapy have been reviewed by Lo Buglio and Neidhart, (1974). Transfer factor as described by Lawrence (1969) differs from immunogenic RNA, also derived from lymphocytes, and which can transfer cellular and humoral immunity (Deckers and Pilch, 1971; Dodd, Scheetz and Rossio, 1973).

## 5. CELLULAR IMMUNITY

Several *in vivo* and *in vitro* assays are available for the demonstration of cell-mediated tumour immunity.

### 5.1 In vivo Assays

#### 5.1.1 Neutralisation tests (Winn assay) and adoptive immunisation

This assay, originally developed by Winn (1959) for studies on

homograft immunity, generally involves inoculation of mixtures of lymphocytes from tumour-immune animals and tumour cells into syngeneic, sub-lethally X-irradiated recipients. This procedure was shown to decrease the rate of growth of methylcholanthrene (MC)-induced sarcomas (Klein et al., 1960; Old et al., 1962). Virus-immune lymphocytes could also protect mice against virally-induced tumours (Sjögren, Hellström and Klein, 1961; Slettenmark and Klein, 1962). Mikulska Smith and Alexander (1966) found that transfer of protection by spleen cells in a rat-sarcoma model was only possible 20 days or more following amputation of the tumour.

Other investigators have been able to adoptively immunise against subsequent tumour challenge by injection of immune lymphoid cells (Old et al., 1962; Delorme and Alexander, 1964). Although somewhat dubious ethically, equivalent experiments have been performed in cancer patients in which autologous leucocytes were mixed with autologous tumour cells and re-injected into the patients' skin. (Southam, 1967). Inhibition of tumour nodule formation was observed in 20 of 44 patients.

#### 5.1.2 Delayed hypersensitivity

Delayed hypersensitivity skin tests have been employed extensively to evaluate the immunocompetence of cancer patients by assessing their reactivity to tumour cell extracts or non-tumour antigens. In one such study using the skin-sensitising hapten dinitrochlorobenzene (DNCB), hypersensitivity to this chemical, shortly after surgery, was correlated with a lower rate of recurrence and improved survival (Pinsky, Wanebo, Mike and Oettgen, 1976a). The use of DNCB as a

therapeutic agent is discussed in a later section.

Autologous skin testing with extracts of a patient's own tumour has been widely investigated and in some studies positive reactions have correlated with remissions or otherwise favourable prognosis (Bluming et al., 1971; Char et al., 1973) but not in others (Gutterman et al., 1973).

## 5.2 In vitro Assays

### 5.2.1 Lymphocyte transformation (blastogenesis)

Assays of lymphocyte transformation provide another way of investigating the cell-mediated response in tumour immunity. Sensitised lymphocytes can be stimulated to form immunoblasts in vitro by exposure to the sensitising antigen. Transformation also takes please without previous sensitisation when lymphocytes are exposed to cells carrying different major histocompatibility antigens (Bain, Vos and Loewenstein, 1964; Dutton, 1965). This effect has meant that in many studies it has been difficult to determine if the observed transformation was due to prior sensitisation to tumour antigens or to a primary immune recognition process. The evidence tends to support the former concept. (Stjernswärd, Vanky and Klein, 1973; Golub et al., 1972). For more information on lymphocyte transformation tests and their associated problems consult Oppenheim and Schecter (1976).

Incubation of patients' lymphocytes with autologous tumour cells or tumour cell extracts have given positive results with many human tumours (Herberman, 1974). In one study cells from lymph nodes

draining patients' tumours were refractory to stimulation, in contrast to the positive reaction of peripheral blood lymphocytes from the same patient (Stjernswärd and Vanky, 1972; Vanky, Stjernswärd, Nilsonne and Sundblad, 1973). This was probably due to inhibition by excess antigen in the node since the lymph node cells could still stimulate autologous peripheral lymphocytes. In another study greater stimulation by autologous tumour cells was obtained in patients with localised disease than in patients with systemic disease (Mavligit, Ambus, Gutterman and Hersh, 1973).

Blastogenesis assays can also be used to detect the presence of blocking factors in patients' sera. Gutterman _et al_., (1973) have shown that serum inhibition of blastogenesis correlated with good prognosis in leukaemia. However, this has not proved to be the case with other tumours where the observation generally reflects extensive disease and poor prognosis (Stjernswärd _et al_., 1973).

### 5.2.2 Inhibition of leucocyte and macrophage migration

Among the lymphokines released by sensitised lymphocytes activated _in vitro_ by the appropriate antigen, is the macrophage (and monocyte) migration inhibitory factor (MIF) which will retard the migration of macrophages and monocytes from capillary tubes (Morley, Wolstencroft and Dumonde, 1973; Rocklin, 1976). This phenomenon has proved to be a very useful _in vitro_ correlate of delayed hypersensitivity and has frequently been used as an assessment of cell-mediated immunity in tumour-bearers (Herberman, 1974).

In animal models, where macrophage populations can be readily obtained from spleen and peritoneal cavity, the test has demonstrated

tumour-specific immunity (Kronman et al., 1969; Vaage, Jones and Brown, 1972). However, in humans, experimenters generally have to use peripheral blood leucocytes as the source of their reactive cells in one of two modifications of the assay. The first is an indirect assay in which blood lymphocytes are cultured with autologous tumour cells or tumour cell extracts and the culture supernatant then tested for the presence of MIF using non-immune guinea pig macrophages (Hilberg, Balcerzak and LoBuglio, 1973; Wolberg and Goelzer, 1971). The other assay involves the direct use, in capillary tubes, of human blood leucocytes as the source for both migratory cells and prducer of MIF, and is more appropriately called the leucocyte migration inhibitory test. This technique has been used to advantage by Cochran and his colleagues (1973) in a study of melanoma and breast carcinoma patients and which showed a correlation between lack of demonstrable MIF and the extent of metastatic spread.

### 5.2.3 Inhibition of leucocyte adherence

Similar to the MIF and LIF assays is the leucocyte adherence inhibition assay in which peripheral blood leucocytes are prevented from adhering to glass by exposure to sensitising antigen (Halliday and Miller, 1972; Maluish and Halliday, 1975). Use of this test in studies of melanoma and breast carcinoma indicated that patients with small tumour burdens (stage I or II), in contrast to those with large tumour burdens (stage III or IV), generally had peripheral leucocytes which exhibited adherence inhibition when exposed to tumour extracts (Grosser and Thomson, 1975; Marti and Thomson, 1976). The

monocyte was shown to be the indicator cell manifesting non-adherence and this reaction was associated with the binding of tumour antigen via specific cytophilic anti-tumour IgG antibody on the monocyte surface (Grosser, Marti, Proctor and Thomson, 1976; Marti, Grosser and Thomson, 1976). Furthermore, the non-reactivity of monocytes in patients with advanced cancer was attributed to the failure of serum anti-tumour antibody to bind to monocytes because of an excess of tumour antigen and immune complexes (Grosser and Thomson, 1976).

### 5.2.4 Colony inhibition and cytotoxicity assays

A number of *in vitro* assays have now been developed for the assessment of cell-mediated immunity to tumours. These assays generally consist of a period of incubation in which tumour cell monolayers in Petri dishes or 96-well microculture plates are exposed to the test lymphoid cells, followed by some assessment of remaining viable tumour cells. Growth inhibition (cytostasis), or death or injury (cytotoxicity) of tumour target cells is determined by the relative effects of normal (non-immune) lymphoid cells and the test (tumour-bearer or tumour-immune) lymphoid cells. As there are a number of reviews and articles dealing with these *in vitro* assays the following descriptions will be brief. (For more details see: Perlmann and Holm, 1969; Cerottini and Brunner, 1974; Hellström and Hellström, 1974; Herberman, 1974; Baldwin, 1975; Herberman and Oldham, 1975; Ting, Park, Nunn and Herberman, 1977).

The various assays differ principally in the way numbers of viable cells are assessed at the end of the test. The earlier assays involved visual counting of cells (Takasugi and Klein, 1970; Hellström

et al., 1971), cell colonies (Hellström, 1967), or of cell nuclei (Roseneauand Moon, 1961; Wilson, 1963; Ulrich and Keiler, 1969). In addition to being tedious, these methods lacked objectivity and have now, therefore, been largely superseded by a number of assays utilising radioisotope cytoplasmic, DNA, or RNA labels such as: sodium ($^{51}$Cr)-chromate (Brunner et al., 1968); ($^{3}$H)-proline (Bean et al., 1973); ($^{3}$H)-thymidine (Jagarlamoody et al., 1971); ($^{14}$C)-thymidine (Vainio et al., 1964); 5-($^{125}$I)iodo-2'-deoxyuridine ($^{125}$IUDR: Cohen et al., 1971); ($^{3}$H)-uridine (Hashimoto and Sudo, 1971); Technitium-99m (Barth et al., 1972). These assays measure either isotope released from dead or injured cells, or the isotope incorporated into viable cells remaining at the end of the test.

Despite being convenient to use, the various radioisotope techniques do have disadvantages. The ($^{51}$Cr) method suffers from a high spontaneous release of the label restricting the assay to short term incubation periods of less than 20 hours. Some nucleic acid labels, particularly ($^{3}$H)-thymidine and ($^{3}$H)-uridine can be reutilised by other viable cells when released. The thymidine labels, including the thymidine analogue ($^{125}$IUDR), can be competitively inhibited by cold thymidine or its derivatives released into the culture system by dead or dying cells (Opitz et al., 1975; Kasahara and Shioiri-Nakano, 1976; Evans and Booth, 1976). The label itself can also be denatured *in vitro* (Marsh and Perry, 1964).

Other problems that have been encountered with microcytoxicity assays in general have been: *in vitro* development of lymphocyte activation and/or suppression; non-specific cytotoxicity; loss of activity of effector cells with prolonged culture; stimulation/feeder

effects of lymphoid cells on target cells; inadequate controls due to 'natural' cytotoxicity or target cell stimulation; and discrepancies in results when using established cell-lines as opposed to short-term cultures of excised tumours (Baldwin, 1975; Herberman and Oldham, 1975; Perlmann, Troye and Pape, 1977). These difficulties, more apparent in human cancer work, require that the chosen assay be thoroughly investigated before it can be deemed reliable for a particular study. References to studies employing in vitro assessment of cell-mediated tumour immunity can be found in the papers and reviews cited above.

### 5.3 Effector cells

#### 5.3.1 Immunoblasts

Immunoblasts, highly mobile, large lymphoid blast cells with cytoplasmic basophilia, pyroninophilia, prominent nucleoli and frequent mitotic figures, appear in antigen-stimulated lymph nodes from which they are subsequently released in large numbers into the blood stream (Crowther, Hamilton Fairley and Sewell, 1969). They contain and secrete antibody, and can be potent cytotoxic effector cells in vitro, the cytotoxicity being mediated by the antibody. Following inoculation of syngeneic tumour cells, immunoblasts are present in the lymph for only a short period. Their reappearance on extirpation of the growing tumour suggests that they are inhibited from leaving local nodes by the tumour presence (Alexander and Hall, 1970). Immunoblasts responding to tumour antigens have been used therapeutically in an experimental system (Delorme and Alexander, 1964).

5.3.2 Lymphocytes

The evidence demonstrating the involvement of lymphocytes in both the initiation and the effector phases of tumour immunity is now considerable, and has been extensively reviewed (Perlmann and Holm, 1969; Baldwin, 1973; Hellström and Hellström, 1974; Cerottini and Brunner, 1974; Herberman, 1974). By using T cell-deprived animals, anti-sera to T cells and macrophages, and various cell separation procedures, it has been possible in these studies to demonstrate dependency for T cells, or for T cells and B cells in tumour immunity. However, proof that these cells can be cytotoxic effector cells *in vitro* and *in vivo* has been more difficult to establish unequivocally.

This is not the case in allograft immunity and in allogeneic tumour immunity where the T cell has been affirmed as an autonomous cytotoxic effector cell *in vitro* (Golstein, Wigzell, Blomgren and Svedmyr, 1972; Golstein and Blomgren, 1973; Lohmann-Matthes and Fischer, 1972). A similar role has also been claimed for T cells *in vivo* (Sprent and Miller 1972; Cerottini and Brunner, 1974) but the difficulty of excluding T cell-macrophage cooperation *in vivo* in such cell transfer experiments necessitates that they only provide evidence of T cell dependency.

Analyses of the cytotoxic effector cells in a number of syngeneic tumour systems have now shown that T cells can be cytotoxic in the absence of other cell types. In rats, cytotoxic cells directed at Gross-virus-induced lymphoma cells were inhibited by anti-theta serum but unaffected by removal of adherent cells and B cells (Djeu *et al*., 1974). Currie and Gage (1973) isolated radiosensitive, non nylon-

adherent (and therefore suggestive of T cells) cytotoxic cells from regional lymph nodes of rats bearing a sarcoma. Leclerc, Gomard, Plata and Levy (1973) have also demonstrated T cell effector cells in the Moloney sarcoma virus (MSV) system. The MSV system has been closely studied by Lamon and his group who found both T cells and B cell dependent non-T cells capable of the *in vitro* killing of cells expressing virally-induced antigen (Lamon, 1974). T cell activity was greatest during the early part of tumour development and gradually disappeared during regression, whereas the non-T cell activity was present throughout the experiment and showed peak activity in regression. This heterogeneity in effector cell populations has also been described in mice bearing syngeneic MC-induced sarcoma (Nelson, 1974). The non-T cell designation has been used above because of the possibility that K cells are present in the T cell-depleted populations (*vide infra*).

Cytotoxic T cell effectors have been observed in a number of human tumour studies (Wybran *et al*., 1973). O'Toole *et al*., (1973) on the other hand only found non-T cell effectors in a study of bladder cancer patients.

### 5.3.3 K cells (antibody-dependent cell-mediated cytotoxicity)

Target cells exposed to specific IgG antibody *in vitro* become susceptible to lysis by non-immune, C3 and Fc receptor-bearing cells. These cells can be macrophages or monocytes (Dennert and Lennox, 1973; Zighelboim *et al*., 1973; Evans, 1975), or killer cells (K cells) (Perlmann and Holm, 1969; Perlmann, Perlmann and Wigzell, 1972; MacLennan, 1972a; Cerottini and Brunner, 1974). The ontogeny of K

cells is still the matter of much debate. K cell-mediated cytotoxicity can be surprisingly efficient with regard to the antibody levels required. Perlmann _et al_., (1972), reported that some sera were active at dilutions of $10^{-9}$ - $10^{-11}$.

Elevated K cell activity has been observed in the spleens and tumour-draining lymph nodes of MC-induced fibrosarcoma-bearing mice (Calder, Irvine and Ghaffar, 1975; Ghaffar, Calder and Irvine, 1976a). Antibody-dependent macrophage-mediated cytotoxicity is discussed in the following section.

### 5.3.4 Macrophages

The free macrophage and the fixed tissue macrophage (histiocyte) represent the final differentiation stages in a cell series which has progressed via bone marrow precursors, promonocytes and monocytes. This cell series, collectively known as the mononuclear phagocyte system (Van Furth, Langevoort and Schaberg, 1975), constitutes an essential part of the reticuloendothelial system (RES) which is involved in particle clearance and degradation. In addition to their scavenging work, mononuclear phagocytes exhibit a multiplicity of functions including wound healing, production and breakdown of biologically important products and participation in immune responses (for details see Van Furth, 1975; Nelson, 1976a).

Macrophage involvement in an immune response can occur at any or all levels; afferent, central or efferent. For example, by phagocytosing and processing antigens they can initiate the primary (Panijel and Cayeux, 1968; Gallily and Feldman, 1967) and sometimes the secondary (Panijel and Cayeux, 1968; Feldman and Palmer, 1971)

antibody responses. Macrophages or their products are involved centrally in the cooperation of B and T cells in their response to antigens, and in lymphocyte activation and immunoregulation (Basten and Mitchell, 1976; Nelson, 1976b). However, it is principally the role of the macrophage as an effector cell which has interested tumour biologists.

*In vitro* studies have consistently shown that macrophages can affect the replication and viability of tumour cells (see reviews by Levy and Wheelock, 1974; Lejeune, 1975; Alexander, 1976; Lohmann-Matthes, 1976; Evans and Alexander, 1976; Keller, 1976a; 1977). The observed anti-tumour effects are dependent on the properties of target cells and effector cells, and on their relative numbers, and may be manifested as promotion of tumour cell growth, cytostasis, or cytolysis (Keller, 1976a; Cullen, unpublished observations). Cytotoxic effects observed at higher effector to target ratios may give way to growth enhancing effects at lower ratios (Keller, 1976a). Effector macrophages may be specifically or non-specifically cytotoxic depending on the manner in which they were activated. Activation is used here to indicate the acquisition or stimulation of cytotoxic or cytostatic potential.

Specifically cytotoxic macrophages may be obtained from the peritoneal cavities of mice immunised with allogeneic cells (Granger and Weiser, 1964, 1966; Den Otter, Evans and Alexander, 1972, or with syngeneic tumour cells (Evans and Alexander, 1970), and also from mice bearing a growing subcutaneous tumour (Evans, 1973). Evidence that macrophages exert an anti-tumour effect *in vivo* came from neutralisation (Winn assay) studies in which immune lymphoid cells were mixed with tumour cells and injected into normal animals. It was found that the

subsequent inhibition of tumour development was mediated by host macrophages and not by the injected lymphocytes (Evans and Alexander, 1970; Zarling and Tevethia, 1973a, b).

This cooperation between normal, non-cytotoxic macrophages and immune lymphocytes to produce macrophage-mediated cytotoxic effects has also been demonstrated _in vitro_ in both syngeneic (Evans and Alexander, 1970) and allogeneic systems (Evans and Alexander, 1972a; Evans and Grant, 1972; Lohmann-Matthes _et al_., 1972; Pels and Den Otter, 1974). The immune lymphocytes were said to "arm" the macrophages which were then specifically cytotoxic for the immunising tumour cells (Evans and Alexander, 1970; 1972a, b). Specific arming of macrophages has also been achieved _in vitro_ by a T cell-dependent supernatant factor released by immune lymphocytes cultured with the sensitising antigen (Evans and Alexander, 1971; Lohmann-Matthes, Ziegler and Fischer, 1973; Pels and Den Otter, 1974).

Macrophages can also bind (via their Fc receptors) and specifically kill antibody-coated tumour cells (Dennert and Lennox, 1973; Zighelboim _et al_., 1973; Evans, 1975; Evans and Alexander, 1976).

Following exposure to their specific target cells armed macrophages become activated and can exert non-specific growth inhibitory effects against a variety of target cells (Evans and Alexander, 1972a). Non-specific cytotoxicity can also be induced in macrophages by a variety of agents some of which, for example, double-stranded RNA and endotoxin, can activate macrophages both _in vivo_ and _in vitro_ (Evans and Alexander, 1976). _In vivo_ activation has also been obtained by administration of killed _Corynebacterium parvum_ (Scott, 1974c), pyran copolymer (Morahan and Kaplan, 1976) and Freund's complete adjuvant (Hibbs, Lambert and Remington, 1972). Of particular interest has been the observation that activated macrophages can be

recovered from animals suffering persistent infection with one of a number of phylogenetically divergent organisms including: Toxoplasma gondii, Besnoitia jellisoni, Listeria monocytogenes (Hibbs et al., 1971; 1972; Krahenbuhl and Remington, 1974); BCG (Hibbs, 1975a, b; Evans et al., 1973; Cleveland, Meltzer and Zbar, 1974); a strain of Pasteurella (Evans and Alexander, 1972a); Nippostrongylus brasiliensis (Keller and Jones, 1971). It should be noted that, as suggested by these observations, studies into the anti-tumour role of macrophages often closely parallels work on the microbicidal effects of activated macrophages (Mackaness and Blanden, 1967; Nelson, 1974; Mackaness, 1976).

Non-specifically cytotoxic macrophages may also be obtained by incubating non-cytotoxic macrophages in the cell-free supernatant from cultures of stimulated lymphocytes. The lymphocytes have been stimulated in a variety of ways including exposure to sensitising tumour antigens (Lohmann-Matthes et al., 1973), tumour unrelated-antigen (Piessens, Churchill and David, 1975), concanavalin A (Fidler, Darnell and Budmen, 1976) or the mixed lymphocyte reaction (Dimitriu et al., 1975). The macrophage activating factor (MAF) is a lymphokine-like molecule (Dumonde et al., 1975) and various investigators have put its size in the range of 35,000-55,000 daltons (Nathan et al., 1973; Lohmann-Matthes, 1976; Dy et al., 1976).

Several groups have observed that non-specifically activated macrophages are capable of a considerable degree of non-immunologic discernment in that they selectively kill or inhibit transformed (malignant) cells (Hibbs, Lambert and Remington, 1972; Hibbs, 1973; Holterman, Klein and Casale, 1973; Piessens et al., 1975; Keller,

1974). However, for reasons as yet unknown, different tumour lines vary in the degree of their susceptibility, and to further complicate the issue, many normal tissue cells can be just as sensitive as transformed cells to macrophage cytotoxicity (Kaplan, Morahan and Regelson, 1974; Keller, 1974, 1975, 1976c; Jones, McBride and Weir, 1975; Nathan and Terry, 1975). It has also been reported that DNA synthesis of transformed lymphocytes can be inhibited by activated macrophages. (For a review on the non-specific immunogrgulatory activities of macrophages see Nelson, 1976b). This complexity may reflect the existence of sub-populations of macrophages having different functions or different degrees of activity (Walker, 1976; Wing, Gardner, Ryning and Remington, 1977). Calderon and Unanue (1975) have described two fractions in cultures of the same peritoneal cell population, one of which has a stimulatory effect and the other an inhibitory effect on cell growth.

Macrophages are endowed with an extensive armoury with which to exert their anti-tumour effects. These weapons may involve cell-to-cell contact (Hibbs, 1974; Keller, 1976a; Piessens, 1977) and/or secretory products (Davies and Allison, 1976). Extracellular killing by labile, lytic factors has been described (Melsom, Sanner and Seljelid, 1975; Sethi and Brandis, 1975). These factors could be complement cleavage products such as C3a, which has been shown to be cytolytic, and C3b which can be involved in secretion of hydrolases (Ferluga _et al_., 1976; Schorlemmer, Davies and Allison, 1976). Recent work showing that macrophage cytotoxicity can be abrogated by protease inhibitors would suggest that proteases are involved in target cell lysis (Hibbs _et al_., 1977).

Many studies have stressed the importance of macrophage-target cell contact for the expression of cytotoxic effects (Evans and Alexander, 1970; Hibbs, 1974; Keller, 1976a; Piessens, 1977). Hibbs (1974) showed that, following cell fusion between target cell and macrophage, lysosomal enzymes were transferred to the target cell which subsequently underwent lysis.

In addition to the cell-transfer experiments of Zarling and Tevethia (1973a,b) described above, a number of other experiments and observations have confirmed the role of macrophages in tumour-host interactions. In the absence of a macrophage-free strain of laboratory animal, macrophage depletion or inactivation must be conducted using a macrophage poison such as carageenan or silica (Keller, 1976b; Levy and Wheelock, 1975). Administration of these agents on the day of tumour inoculation significantly enhanced tumour growth and, since the lysosome stabilising agent polyvinylpyridine N-oxide (PVNO) neutralised this effect, a role for macrophages in host defence against tumour implantation was assumed (Keller, 1976b). However, the cellular and biochemical consequences of the administration of silica and carageenan may offer alternative explanations (Levy and Wheelock, 1975; Keller, 1976b). The observed effects may have been due to release from lysed macrophages of growth promoting substances or of factors which interfered with lymphoid cell function (Keller, 1976b).

The anti-inflammatory agent sodium aurothiomalate has also been utilised in studies of macrophage anti-tumour effects (McBride et al., 1975b; James et al., 1976; Ghaffar et al., 1976b). This compound inhibits lysosomal enzymes (Ennis et al., 1968) and suppresses

phagocytosis and migration of macrophages (Jessop et al., 1973; Vernon-Robert et al., 1973).

Further information on the anti-tumour role of macrophages has derived from studies on the macrophage population of human and experimental tumours. As mentioned earlier, infiltrating macrophages can account for a considerable proportion of a tumour's bulk. This has been demonstrated by disaggregating tumours enzymatically and identifying macrophages by a variety of parameters (Evans, 1972, 1973; Pross and Kerbel, 1976; Szymaniec and James, 1976; Kerbel and Pross, 1976; K. Moorre and Moore, 1977; M. Moore and Moore, 1977). The capacity of a tumour to metastasise was shown to be inversely proportional to its macrophage content in experimental tumours (Eccles and Alexander, 1974) and, although only a preliminary survey, also in some human tumours (Gauci and Alexander, 1975). It has also been shown that the number of host infiltrating cells is directly proportional to the immunogenicity of a particular tumour (Evans, 1972; K. Moore and Moore, 1977; M. Moore and Moore, 1977). Tumour-derived macrophages have been shown, in some studies, to be cytotoxic (Evans, 1973, 1975; Haskill et al., 1975, 1976). Furthermore, macrophages are more numerous and more cytotoxic in regressing Moloney sarcoma than in progressing tumours (Russell, Doe and Cochrane, 1976; Russell and McIntosh, 1977). These studies thus provide strong corroboration of a significant role for macrophages in the host's defence against tumour growth. Why such defences should often prove inadequate is still largely a mystery. This paradox will be returned to in the discussion on escape mechanisms.

5.3.5 Other effector cells

Besides B cells and T cells, a third heterogeneous group of lymphoid cells (variously called null cells, N cells, nonselective cytotoxic cells, or natural killer (NK) cells), have attracted considerable interest as effectors of 'spontaneous' cell-mediated cytotoxicity to a number of tumours (Kiessling, Klein and Wigzell, 1975; Herberman, Nunn and Lavrin, 1975; Kiuchi and Takasugi, 1976). These cells appear to lack the conventional markers for T or B cells, are non-adherent and non-phagocytic. Natural killer cell activity has been associated with a cell population mediating K cell activity in some human studies (Kalden, Peter, Roubin, and Cesarrini, 1977) but not in mice (Kiessling et al., 1976; Herberman et al., 1975).

In addition a subpopulation of adherent peritoneal cells has been isolated which is poorly phagocytic and which appears to account quantitatively for the tumour inhibitory activity of the total cell population from which they were derived (Nathan, Hill and Terry, 1976). These cells were present in untreated mice although BCG treatment greatly increased their number (Nathan et al., 1976). These cells may be a subpopulation of B cells (Nathan, Asofsky and Terry, 1977).

The non-specific effects of natural killer cells and macrophages point to the existence of a primitive surveillance system which possibly pre-dates the evolution of the vertebrate immune system as it is now known (Holterman et al., 1976; Keller, 1977).

Another cell population with the demonstrated anti-tumour effects is the neutrophil (Pickaver, Ratcliffe, Williams and Smith, 1972).

## 6. IMMUNOLOGICAL ESCAPE OF TUMOURS

The preceding sections have described the battery of host responses that can be demonstrated to act against tumour cells *in vitro* and *in vivo* but which are often powerless to reject the growing tumour. This paradox, perhaps best illustrated by the presence within many tumours of cytotoxic host cells and by the phenomenon of concomitant immunity, in which tumour-bearing animals may reject a challenge with the same tumour in another site (Klein *et al*., 1960). Such enigmas have prompted discussion and explanation by many authors (Klein, 1969, 1975; Woodruff, 1973; Jeejeebhoy, 1975).

One explanation might be that the tumour may only be weakly immunogenic or even non-immunogenic. This could occur through immunological selection pressures on tumour cells giving rise to less immunogenic clones. Two possible mechanisms for such phenotypic modification of tumour cells are: antigenic modulation as described by Boyse and Old (1969) for the thymus leukaemia (TL) antigen in mice, and the shedding of antigens from the cell surface (Currie and Alexander, 1974; Davey, Currie and Alexander, 1976).

Another possibility is that the tumour cells "sneak through" the host defences. This concept arose from the observation that a small or large tumour cell inoculum would grow, whereas an intermediate cell dose was rejected (Old, Boyse, Clarke and Carswell, 1962). An alternative, perhaps not unrelated to this sneaking through effect, is that a weak immune response could actually promote tumour growth. Attention was drawn to this phenomenon by Prehn who called it immunostimulation (Prehn and Lappé, 1971; Prehn, 1972), and it has since been demonstrated in both *in vitro* and *in vivo* systems by a

number of workers (Fidler, 1973; Medina and Heppner, 1973; Jeejeebhoy, 1974; Kall and Hellström, 1975).

However, the explanation of this failure of anti-tumour defences that has received most attention is that factors derived from the tumour or the host interfere directly, or via suppressor cells, to inhibit, or block the response. It has been known for most of this century that prior injection of killed tumour cells, or tumour extracts with the aim of eliciting protection against subsequent live tumour challenge, frequently gives the opposite result, namely, enhancement or facilitation of tumour growth (Snell _et al_., 1946; Voisin, 1971). It was found that enhancement could be transferred by serum and that the active agent was associated with immunoglobulins (Kaliss and Kandktsch, 1956).

The Hellströms later showed that serum from tumour-bearing individuals could specifically abrogate the _in vitro_ cytotoxic effects of lymphoid cells toward the appropriate target cells (I. Hellström and Hellström, 1969; K.E. Hellström and Hellström, 1970, 1974, 1976). This phenomenon has since been observed in many animal and human tumour systems (Sjögren _et al_., 1971; Baldwin, Price and Robins, 1973; Plata and Levy, 1974; Blair and Lane, 1974). Blocking effects by tumour-bearer serum have been noted in other assay systems such as leucocyte adherence test (Grosser and Thomson, 1976) and the lymphocyte transformation test (Stjernswärd _et al_., 1973).

These serum blocking factors have now been shown to be antigen-antibody complexes (Sjögren _et al_., 1971; Baldwin, Price and Robins, 1972b; Jose and Seshadri, 1974) which mediate their effects either at the tumour cell surface by masking surface antigens, altering their

distribution or causing their internalisation (Leonard, 1973), or by binding directly to the effector cells via Fc receptors (Baldwin et al., 1973; Blair and Lane, 1974; Gorczynski et al., 1975; Plata and Levy, 1974). Inhibition of effector cell activity can also be mediated by tumour antigen alone (Plata and Levy, 1974; Baldwin et al., 1973; Vaage, 1973). The development of tumour enhancement following administration of tumour antigen obviously has grave implications for those clinicians using immunotherapy schedules involving such a procedure.

Complimentary to these studies are those demonstrating that blocking effects can be neutralised by the addition of excess antibody (I. Hellström and Hellström, 1970; Bansal and Sjögren, 1971; Robins and Baldwin, 1974). This effect would suggest that therapy comprising the passive transfer of anti-tumour antibodies might be beneficial.

In addition to antigen and immune complexes various other factors with immunosuppressive activity have been found in the sera of cancer patients and tumour-bearing animals. For example, in association with certain tumours increased levels can occur of alpha foetoprotein (Murgita and Tomasi, 1975a, b) and alpha-2-globulins which have immunosuppressive properties (Cooperband et al., 1976; Glaser and Nelkin, 1972). The active principle of the alpha-2-globulin factor has been shown to be a peptide of molecular weight, 6,000 - 8,000 Daltons (Occhino et al., 1973). Small molecular weight immunosuppressive factors have also been found in tumour-bearers by other workers (Nimberg et al., 1975; Yamazaki et al., 1973).

The origin of these suppressive factors has not always been clear.

Macrophages, for example, can produce a range of immunoregulatory products (Nelson, 1976b). Viruses or mycoplasma carried by tumour cells, or extraneous bacterial contamination can also lead to enhancement (Kamo, Patel and Friedman, 1976), or suppression of immune responses (Kateley, Kamo, Kaplan and Friedman, 1974; Bonnard *et al.*, 1976; Kampschmidt and Schultz, 1963). However, there is evidence from cell culture studies that tumour cells *per se* do release immunosuppressive factors (Werner *et al.*, 1973; Fauve *et al.*, 1974; Wong, Mankovitz and Kennedy, 1974). It should also be noted that some tumours can evidently produce prostaglandins, another group of immuno-regulatory agents (Plescia *et al.*, 1975; Sykes and Maddox, 1972).

The detailed mechanisms by which these blocking factors mediate immunosuppression are not yet known, but it seems likely that some, particularly antigen or antigen-antibody complexes, operate through the suppressor cell system. Cells capable of suppressing the immune reactivity have been found in many tumour systems (Gershon, 1974; Treves *et al.*, 1974; Kolsch, Stumpf and Weber, 1975; Fujimoto *et al.*, 1976 a,b). The suppression may be antigen specific (Fujimoto *et al.*, 1976a,b) or non-specific (Kirchner *et al.*, 1974a,b; 1975; 1976). Suppressor cells have been identified as T cells (Folch and Waksman, 1974a,b; Fujimoto *et al.*, 1976a,b), macrophages (Kirchner *et al.*, 1976), or B cells (Gorczynski, 1974). Macrophage-mediated suppression may in fact be the result of a T cell signal cytophilic for macrophages (Taylor and Basten, 1976).

In addition to the suppressor cell system, tumour-associated or tumour-derived suppressive factors could affect directly the performance of effector T cells, K cells and macrophages by blocking Fc receptors, or receptors for immunoregulatory signals. For comprehensive reviews of tumour-mediated immunosuppression see James

(1977) and Kamo and Friedman (1977).

## 7. IMMUNOTHERAPY

Success of the conventional cancer treatments, surgery and radiotherapy, are subject to certain limitations. Surgical excision may prove impossible if the tumour has originated in, or spread to, a vital organ, and radiotherapy is limited by the relative radiosensitivity of the tumour tissues. In addition, both therapies can be immunosuppressive. For example, depressed *in vitro* lymphocyte blastogenic responses to mitogens have been observed following surgery (Park *et al*., 1971) and anaesthesia (Bruce and Wingard, 1971). The immunosuppressive effects of whole-body irradiation of animals is well documented (Micklem and Loutet, 1966; Anderson and Warner, 1976), and in man even localised irradiation, for example of the chest wall in breast cancer, can result in prolonged depression of blood lymphocyte levels (Stjernswärd *et al*., 1972). However, both surgery and radiotherapy have a more serious limitation in that they are often powerless to deal with tumours which have already metastasised. Disseminated cancer thus requires a systemic approach to treatment. Chemotherapy and immunotherapy are, potentially at least, capable of fulfilling this role.

To be successful an anti-cancer drug must exhibit a differential cytotoxicity, at the dose used, between tumour cells and normal tissue, and this generally means exploiting differences in metabolism or cell kinetics. An example of the former mode is the use of L-asparaginase in the treatment of leukaemia (Beard *et al*., 1970). Unfortunately, the value of this treatment is limited by the development of resistant

cells with asparagine synthetase activity, possibly due to gene de-repression in response to the presence of asparaginase (Haskell and Cannellos, 1970). Where the difference in drug sensitivity (the therapeutic index) has been sufficiently high, chemotherapy has been strikingly successful, particularly in the treatment of Burkitt's lymphoma (Burkitt and Wright, 1970), acute lymphoblastic leukaemia, Hodgkin's disease and choriocarcinoma (McElwain, 1976). Many anti-cancer drugs act through an interference of cell division and thus, unfortunately, host cells, such as bone marrow cells and lymphoid tissue, with high rates of proliferation will also be affected, leading to immunosuppression (Hersh and Freireich, 1968).

Absence of target selectivity in the available chemotherapeutic drugs has led to a resurgence of interest in immunotherapy which should, in theory, provide the desired specificity. To be successful any immunotherapeutic procedure must potentiate specific and/or non-specific immune responses to the tumour cells in order to overcome the various escape mechanisms outlined in section 6 above. The modes of immunotherapy outlined below have been developed during the past 100 years and have been employed alone or in combination with surgery, radiotherapy or chemotherapy. An overview of early work in this field may be gleaned from articles by Snell _et al_., (1946); Nauts, Swift and Coley (1946); Southam (1961); Voisin (1971); Currie (1972); and Gutterman _et al_., (1974).

## 7.1 Specific active immunotherapy

This involves injection of inactivated autologous tumour cells or tumour extracts (Bloom _et al_., 1973; Currie, 1973; Ikonopisov _et al_.,

1970). This method carries the possible hazard of enhancement (*vide supra*). A modification of this form of therapy involves increasing the immunogenicity of tumour cells by treating them with neuraminidase (Currie and Bagshawe, 1969; Simmons *et al*., 1971) or by coupling various agents such as xenogeneic globulins to them (Czajkowski *et al*., 1967).

## 7.2 Specific passive immunotherapy

Specific passive immunotherapy is achieved by the transfer of immune syngeneic, allogeneic or xenogeneic serum (Murray, 1958; Bansal and Sjögren, 1972). In addition to possible cytotoxic effects on the tumour cells, this therapy mode could have the advantage of counteracting serum blocking factors (*vide supra*). Anti-tumour immunoglobulins have also been used as carriers of other therapeutic agents. For example, melanoma patients treated with chlorambucil bound to goat or rabbit anti-human melanoma globulins had prolonged survival when compared with patients on either chemotherapy or antiserum alone (Ghose *et al*., 1972, 1975, 1977).

## 7.3 Specific adoptive therapy

Specific adoptive therapy using immune lymphoid cells has also been investigated (Nadler and Moore, 1969). This trial involved cross-immunisation of pairs of patients with tumour, and thus lymphocytes subsequently exchanged would also recognise HLA antigens. This technique has recently been given new impetus with the development of methods of *in vitro* sensitisation of lymphoid cells to tumour

cells (K.E. Hellström and Hellström, 1976; Treves, Cohen, Schecter and Feldman, 1976).

An alternative to the use of lymphocytes is to transfer lymphocyte derived mediators of immunity such as transfer factor or immunogenic RNA (Lawrence, 1969; Lo Buglio and Neidhart, 1974), or lymphokine preparations (Papermaster et al., 1976). The lymphokine preparation was used to treat cutaneous metastatic lesions and seems to operate in a similar way to DNCB when used as an anti-tumour agent. DNCB, a skin sensitising agent, is used to induce delayed hypersensitivity reactions in the vicinity of neoplastic skin lesions resulting in an inflammatory response in the tumour, with subsequent regression (Klein and Holterman, 1972).

### 7.4 Non-specific immunotherapy

Non-specific anti-tumour effects using a large number of varied biological and chemical agents have now been claimed. It was known in the nineteenth century that tumour regressions sometimes followed acute bacterial infection, particularly erysipelas (Coley, 1891) and this formed the basis of attempts at deliberate infection with *Streptococci* in cancer patients (Coley, 1893). Coley and others eventually progressed to the use of killed preparations of mixtures of various bacteria which became known as Coley's toxins (reviewed by Nauts, Swift and Coley, 1946). A lyophilised preparation of *Streptococcus haemolyticus* (OK-432) has been shown to inhibit the growth of transplanted tumours (Okamoto et al., 1966; Ishi, Yamaoka, Toh and Kikuchi, 1976).

Other micro-organisms with reported anti-tumour properties include Bordetella pertussis (Likhite, 1974); Bacillus Calmette-Guérin (BCG) or its methanolic extraction residue - MER (Weiss, Bonhag and De Ome, 1967; Old, Clarke and Benacerraf, 1959; Zbar, Bernstein, Tanaka and Rapp, 1970); anaerobic corynebacteria such as Corynebacterium parvum (Woodruff and Boak, 1966; Scott, 1974c; Milas, Hunter and Withers, 1974b; Ghaffar, Cullen, Dunbar and Woodruff, 1974); Listeria monocytogenes, Toxoplasma gondii, Besnoitia jellisoni (Hibbs et al., 1971, 1972; Krahenbuhl and Remington, 1974); spores from non-pathogenic Clostridia species (Möse and Möse, 1964; Thiele, Arison and Boxer, 1964). In addition, there are a number of preparations of yeast or fungal origin-lentinan, zymosan, pseudo-nigeran - which contain anti-tumour glucose polymers - glucans (Bradner, Clarke and Stock, 1958; Chihara et al., 1969, 1970; Bomford and Moreno, 1977; Cook et al., 1977). Many micro-organisms also produce metabolites (e.g. L-asparaginase, neuraminidase), antibiotics and endotoxins which could have anti-tumour effects (see review by Rosenkranz, 1973).

Anti-tumour effects have also been demonstrated using natural or synthetic polyanions such as double-stranded RNA or Polyinosinic-Polycytidylic acid (Poly I-C) (Parr, Wheeler and Alexander, 1973) and Pyran - a copolymer of divinyl ether and maleic anhydride (Morahan and Kaplan, 1976; Schultz et al., 1977b).

The antihelminthic drug levamisole has aroused a great deal of interest in oncologists because of its ability to augment immuno-logical functions and would thus seem to be of value as supportive therapy in various treatment schedules, including those using chemo-

therapy or agents such as *C. parvum* (Symoens and Rosenthal, 1977).

Although the mechanism of action of many of these agents is not known in detail, it is probable that some act through a stimulation of the mononuclear phagocytic system and potentiation of immune responses. In some cases, for example BCG and *C. parvum*, both specific and non-specific immune stimulation can occur. Potentiation of specific responses can reflect the existence of antigenic determinants common to both microorganisms and tumour cell. Further details on the anti-tumour effects of BCG and *C. parvum* and on the mechanisms underlying these effects are discussed below.

### 7.5 Combination therapy

Apart from a few isolated and anecdotal cases, immunotherapy has generally proved unsuccessful in treating cancer in man. The success obtained in animal models is perhaps misleading since cures can often be achieved in these systems using conventional therapies. However, despite the present scepticism of the value of immunotherapy many workers remain enthusiastic about its potential, particularly for those regimens using immunopotentiators such as BCG and *C. parvum* (Gutterman, 1977; Weiss, 1977). Judgement ought to await the outcome of the numerous, worldwide, ongoing trials which are using immunological manipulation.

It is now recognised that immunotherapy is most likely to succeed when the tumour load is very small and consequently this mode of therapy is best employed together with substantial reduction of the tumour mass by using surgery, radiotherapy and chemotherapy. Maintenance following cytoreduction can be achieved with a combination of

drug therapy and immunotherapy. Such combined attacks on tumours have led to encouraging results in animal models and in man. For example, Haddow and Alexander, (1964) showed that treatment with autologous tumour cells led to radio-sensitisation of growing rat fibrosarcomas. Glynn et al. (1969) found that a combination of adoptive immunotherapy with cyclophosphamide was much more effective than either treatment alone in treating Moloney-virus lymphomas.

Many combination trials have used, or are using, BCG or C. parvum. The first successful clinical trial employing BCG was that of Mathé and his group who reported that immunotherapy (BCG and/or vaccination from a pool of inactivated leukaemia cells) following intensive cytoreduction significantly prolonged the disease-free interval compared to patients untreated after cytoreduction (Mathé et al., 1969). In contrast, two other trials using BCG therapy for the maintenance of remission have given negative results (Medical Research Council, 1971; Heyn et al., 1973). However, the protocol in these trials did not employ the cytoreduction techniques as used in the Mathé study. It has also been shown that different strains of BCG can have different anti-tumour activities (Hawrylko and Mackaness, 1973; Fortner, Hanna and Coggin, 1974).

Studies in other tumour systems have also claimed improvement using BCG. Morton et al. (1970) showed that intralesional inoculation of BCG caused regression of melanoma skin nodules. Gutterman et al. (1976) reported that combination treatment schedules using BCG and chemotherapy could prolong remission and survival of patients with melanoma, breast cancer and acute leukaemia. Favourable results using BCG in melanoma patients have also been reported by Ikonopisov

(1972) and Spitler, Levin and Wybran (1976). However, negative results have been obtained by Pinsky _et al_. (1976b), and in another study using BCG together with autologous irradiated tumour cells, the immunotherapy actually resulted in acceleration of recurrence (McIllmurray _et al_., 1977).

Assessment of BCG therapy has proved encouraging for a number of other tumours: lung cancer (McKneally _et al_., 1976; Pouillart _et al_., 1976; Holmes _et al_., 1977) and in cancer of the colon and rectum (Mavligit _et al_., 1976).

The methanol extraction residue (MER) of BCG, which has the advantage over BCG of being a non-viable vaccine, has featured in several tumour studies and shown to be of therapeutic value in animals (Minden, Wainberg and Weiss, 1974; Cohen _et al_., 1970) and in man (Moertel _et al_., 1975; Robinson _et al_., 1975). See also articles by Weiss (1977) and Ribi _et al_. (1976).

The only extensive clinical trials using _C. parvum_ reported on to date have been those of Israel and his associates (Israel, 1973, 1975; Israel and Halpern, 1972; Israel _et al_., 1975). These reports claim beneficial effects from the use of _C. parvum_. Several preliminary phase I studies have recently been published which provide information on dose tolerance, immune responsiveness, serum Ig levels and side effects (Fisher _et al_., 1976a; Cheng _et al_., 1976; Minton _et al_., 1976; Woodruff _et al_., 1975; James _et al_., 1975).

## 8. MECHANISMS UNDERLYING THE ANTI-TUMOUR EFFECTS OF BCG AND C. PARVUM

Although BCG and _C. parvum_ are at present the most widely used and studied of the many 'non-specific' immunotherapeutic agents, the

mechanisms underlying their anti-tumour effects have not been completely analysed. The following section provides a synopsis of some of the existing knowledge.

8.1 <u>BCG</u>

The anti-tumour effects of BCG therapy have been observed in animals with established tumours (Nathanson, 1972; Zbar, Bernstein, Tanaka and Rapp, 1970; Zbar and Tanaka, 1971) and in animals receiving tumour transplants following BCG treatment (Old, Clarke and Benacerraf, 1959; Lemonde <u>et al</u>., 1971; Weiss, Bonhag and Leslie, 1966). Prophylaxis with BCG has even extended to reduction in the incidence of radiation or carcinogen induced malignancies (Nilsson, Révész and Stjernswärd, 1965; Piessens <u>et al</u>., 1971). However, it should be noted that BCG is not always effective against tumours (Eilber, Holmes and Morton, 1971; Lemonde <u>et al</u>., 1971; Baldwin and Pimm, 1973a; Old <u>et al</u>., 1961) and can even facilitate their growth (Bansal and Sjögren, 1973; Old <u>et al</u>., 1961; McIllmurray <u>et al</u>., 1977).

BCG appears to be most effective when in contact with the tumour cells. Thus tumour cells mixed with BCG fail to form tumour nodules when injected (Zbar <u>et al</u>., 1970; Bartlett, Zbar and Rapp, 1972; Baldwin and Pimm, 1973b) and established tumours regress when BCG is injected into the tumour (Zbar <u>et al</u>., 1972; Zbar, Ribi and Rapp, 1973; Nathanson, 1972; Zbar and Tanaka, 1971).

The success of BCG therapy is dependent on the immunogenicity of the tumour, strongly immunogenic tumours being most strongly inhibited (Baldwin and Pimm, 1973b).

Other factors determining the outcome of BCG theray are the

method of preparation, dose, and strain of BCG used. Differences in the virulence and anti-tumour effectiveness of various BCG strains have been reported (Hawrylko and Mackaness, 1973; Mathé, Halle-Pannenko and Bourut, 1973; Fortner, Hanna and Coggin, 1974; Mackaness, Auclair and Lagrange, 1973).

In addition to the anti-tumour effects, BCG administration can have a number of consequences such as stimulation of the reticulo-endothelial system (Biozzi, Stiffel, Halpern and Mouton, 1960; Old et al., 1961; Balner, Old and Clarke, 1961), increased non-specific resistance to other bacterial infection (Blanden, Lefford and Mackaness, 1969; Dubos and Schaedler, 1957), accelerated graft rejection (Balner, Old and Clarke, 1962; Vitale and Allegretti, 1963), and stimulation of humoral and cellular immunity to antigenic challenge (Miller, Mackaness and Lagrange, 1973).

Many of these effects may be associated with, or mediated by, BCG-activated macrophages. In addition to the general stimulation of the RES, the macrophage functions of chemotaxis, pinocytosis and random migration are elevated in BCG treated animals (Poplack, Sher, Chaparas and Blaese, 1976; Meltzer, Jones and Boetcher, 1975). Bone marrow macrophage colony formation is also transiently enhanced by BCG inoculation in mice (Fisher et al., 1974).

The significance of macrophages in the anti-tumour action of BCG has emerged from several other lines of research. Macrophages, cytotoxic for tumour cells in vitro have been isolated from BCG-treated animals (Cleveland, Meltzer and Zbar, 1974; Hibbs, 1975a, b). Hibbs (1974) has demonstrated that macrophages activated by BCG kill tumour target cells in vitro by transfer of lysosomes into the tumour cells following cell-to-cell contact. Circumstantial

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evidence that macrophages may be involved in the anti-tumour effects comes from the observation that BCG is effective in athymic nude mice (Pimm and Baldwin, 1975) and in T cell-deprived rats (Moore, Lawrence and Nisbet, 1975, 1976). These results are in contrast to others in which BCG-mediated anti-tumour effects required the development of a T cell-dependent immune reaction to BCG (Zbar, Bernstein and Rapp, 1971; Bartlett, Zbar and Rapp, 1972; Chung, Zbar and Rapp, 1973).

Macrophages may not be the only anti-tumour effector cells mediating BCG's effects. T cells, K cells, B cells and natural killer (NK) cells may all play a part. A recent report describes increased NK cell activity following BCG administration (Wolfe, Tracey and Henney, 1976; see also Parr, 1976).

In experiments where BCG is given intralesionally or together with irradiated tumour cells, specific anti-tumour immunity may be augmented (Hawrylko, 1975; Hanna _et al_., 1973; Scott and Bomford, 1976). This effect and the observation that some tumours share common antigens with mycobacteria (Borsos and Rapp, 1973; Minden, McClatchy, Wainberg and Weiss, 1974; Bucana and Hanna, 1974; Minden, Sharpton and McClatchy, 1976) indicates that in some situations BCG can be considered to be a specific as well as a non-specific agent. Note also that lymphocytes cytotoxic to human tumour cells _in vitro_ were obtained by _in vitro_ culture of normal lymphocytes with various bacterial extracts (Sharma _et al_., 1977). The design of this experiment was such that lymphocytes could have been responding to histocompatability antigens on the tumour target cells and consequently a syngeneic model will be required for confirmation of the result.

8.2 C. parvum

8.2.1 Following the demonstration of marked RES stimulation by a killed vaccine of C. parvum (Halpern et al., 1964), and in view of the known anti-tumour effects of other RES stimulants such as BCG, Woodruff and Boak (1966) examined the effect of a single intravenous (i.v.) injection of C. parvum vaccine on the growth of syngeneic tumour transplants. The treatment significantly delayed appearance of tumour nodules. The value of C. parvum and that of another strain, Corynebacterium granulosum, as anti-tumour agents has since been repeatedly affirmed both in a variety of animal tumour systems (Halpern et al., 1966; Milas et al., 1974a; Fisher et al., 1970; Ghaffar et al., 1974; Scott, 1974c; Olivotto and Bomford, 1974; Mazurek et al., 1976; Bast and Bast, 1976) and in man (Israel and Halpern, 1972; Israel, 1976). In the animal experiments C. parvum administered before or after tumour transplant generally resulted in a delay in the appearance of tumour, retardation of tumour growth and prolongation of survival.

Systemic protection against tumour challenge could be obtained with C. parvum given by the intravenous or intraperitoneal (i.p.) routes but not by the subcutaneous (s.c.) route (Woodruff and Inchley, 1971; Woodruff, Inchley and Dunbar, 1972; Scott, 1974c). Subcutaneous or intradermal application of C. parvum resulted in marked growth inhibition provided that the injection was sufficiently near the tumour site to engage the draining lymph node (Likhite and Halpern, 1974; Scott, 1974b; Woodruff and Dunbar, 1975). Systemic administration of C. parvum and C. granulosum has, in one model, led to complete regression of artificial pulmonary fibrosarcoma metastases

(Milas et al., 1974a,c; 1975a) but this is a rare observation and is not generally found in other models (Scott, 1974c). Lasting regressions however, do often follow administration of C. parvum directly into the tumour (Likhite and Halpern, 1974; Paslin et al., 1974; Scott, 1974b, Milas et al., 1975a).

Promotion of tumour growth as a result of C. parvum therapy has only rarely been observed and only under particular circumstances. Bomford (1977) has recently identified several situations which can result in tumour enhancement by C. parvum. Intravenous C. parvum given before low doses (around the TD50) of tumour given subcutaneously led to enhanced growth. This promotion effect increased with higher doses of C. parvum. Enhancement of leukaemic 1210 in allogeneic mice by C. parvum therapy has also been reported (Mantovani et al., 1976; Berd and Mitchell, 1976). This effect was associated with suppression of antibody-mediated and cell-mediated cytotoxicity (Berd and Mitchell, 1976). In another study using two lymphomas which regress spontaneously in normal mice, regression was abrogated by administration of C. parvum (Woodruff and Warner, 1977).

### 8.2.2 Combined therapy

Combination of C. parvum therapy with chemotherapy (Currie and Bagshawe, 1970; Woodruff and Dunbar, 1973; Fisher et al., 1975), radiotherapy (Milas et al., 1975b; Suit et al., 1975) or surgery (Proctor et al., 1973) resulted in anti-tumour reactions which were more effective than any individual treatment.

A number of specific active therapy modalities have been investigated but with varying success. Subcutaneous injection of X-irradi-

ated cells together with C. parvum evokes a strong protective effect against subsequent tumour challenge (Scott, 1975). In contrast, administration of C. parvum several days before immunisation with irradiated tumour cells abolished the protection normally afforded, by the immunisation procedure (Smith and Scott, 1972; Woodruff, Ghaffar, Dunbar and Whitehead, 1976a). No improvement followed the use of combinations of C. parvum therapy and irradiated tumour cells in other tumour systems (Likhite and Halpern, 1973; Scott, 1974a; Proctor et al., 1973). Synergistic anti-tumour combinations have been reported using C. parvum together with neuraminidase-treated cells (Woodruff and Dunbar, 1973) or cells coated with heterospecific anti-tumour globulin (Woodruff and Inchley, 1971; Woodruff, Inchley and Dunbar, 1972).

### 8.2.3 Toxicity

A number of authors have recorded toxicity using i.v. or i.p. C. parvum in both high (Woodruff and Dunbar, 1973) and routinely used doses (Scott, 1974a; Fisher et al., 1970; Milas, Hunter and Withers, 1974b). Several phase I human cancer studies have reported side effects such as pyrexia and chills, vomiting and headache occurring shortly after or during intravenous infusion with C. parvum (Woodruff et al., 1975; Fisher et al., 1976a, Cheng et al., 1976; Minton et al., 1976). Earlier animal experiments tended to use doses which are now regarded as being large and so consequently carried a greater risk of toxic effects. More recent studies tend to use smaller doses which also reflect more accurately the doses used in humans. Scott and Warner (1976) have shown that weekly small doses of i.v. injected

*C. parvum* gave a good anti-tumour effect but without suppression of T cell function (*vide infra*).

### 8.2.4 Stimulation of mononuclear phagocytes

Several aspects of cellular and humoral immunity are affected by the administration of *C. parvum*. Intravenous or intraperitoneal injection of *C. parvum* or *C. granulosum* results in a powerful stimulation of the RES with consequent hepatosplenomegaly, increased lung weight, increased lymph node weight, and rapid clearance of particulate matter from the blood stream (Halpern *et al*., 1964 Adlam and Scott, 1973; O'Neill, Henderson and White, 1973; Milas, Basic, Kogelnik and Withers, 1975c). Macrophages from *C. parvum*-treated animals exhibit the biochemical and morphological features of activation (Wilkinson *et al*., 1973; Olivotto and Bomford, 1974; Adams, Biesecker and Koss, 1973). A consequence of the increased macrophage activity is the improved resistance of animals to infection (Adlam, Broughton and Scott, 1972; Collins and Scott, 1974).

A lipid fraction isolated from *C. parvum* is chemotactic for macrophages and appears to account for the early activation of macrophages as assessed by carbon clearance (Russell, McInroy, Wilkinson and White, 1976; Otu, Russell and White, 1977). Anti-tumour activity may be due in part to the lipid fraction (McBride, Dawes and Tuach, 1976).

Another early effect of *C. parvum* administration is a stimulation of bone marrow macrophage colony formation (Wolmark, Levine and Fisher, 1974; Baum and Breese, 1976).

8.2.5 Adjuvant activity

*C. parvum* administration to mice and men increases the serum levels of certain immunoglobulin classes and subclasses including antibodies to tumour (James *et al*., 1975, 1976, 1977). Under appropriate conditions antibody responses to both T cell dependent and T cell independent antigens can be augmented (Howard, Christie and Scott, 1973; Warr and James, 1975; Sljivic and Watson, 1977). *C. parvum* has also been shown to be mitogenic for B cells (Zola, 1975). Both adjuvant and mitogenic activities required the participation of macrophages. A phenomenon which is probably related to adjuvanticity in *C. parvum*-treated animals is that of sequestration and retention of lymphocytes in nodes (lymphocyte trapping) (Frost and Lance, 1973).

8.2.6 Effects on cellular immunity

Systemic administration of *C. parvum* depresses many T cell-mediated phenomena such as the graft-versus-host reaction (Howard *et al*., 1967; Scott, 1972a); delayed hypersensitivity (Allwood and Asherson, 1972; Scott, 1974d); homograft reaction (Castro, 1974a; Milas *et al*., 1975c); phytohaemagglutinin-responsiveness and the mixed lymphocyte reaction (Scott, 1972a). In addition, infection with *Trichinella spiralis* may be enhanced by *C. parvum* treatment (Ruitenberg and Steerenberg, 1973). These immunosuppressive features of *C. parvum* appear to be mediated by macrophages (Scott, 1972b; Kirchner, Holden and Herberman, 1975). Local administration of *C. parvum* does not appear to cause systemic immunosuppression (Scott, 1974c).

8.2.7 Anti-tumour mechanisms

The non-specific aspect of systemically administered C. parvum appears to be effected by macrophages. This conclusion is based principally on the observed general stimulation of macrophage activity (*vide supra*) and macrophage cytotoxicity towards tumour cells *in vitro* (Basic *et al*., 1975; Olivotto and Bomford, 1974; Ghaffar *et al*., 1974, 1975) following C. parvum injection. The importance of the macrophage as effector cell is further supported by the observation that systemic C. parvum is also effective against tumours in T cell-deprived mice (Woodruff, Dunbar and Ghaffar, 1973; Scott, 1974a), in athymic nude mice (Woodruff and Warner, 1977) and in ALS-treated mice (Castro, 1974b). Cytotoxic macrophages have been recovered from T cell-deprived and nude mice treated systemically with C. parvum (Ghaffar *et al*., 1975). On the other hand, the marked anti-tumour effect usually manifested by local application of C. parvum does not appear in T cell-deprived and nude mice (Scott, 1974b; Woodruff and Dunbar, 1975; Woodruff and Warner, 1977).

Strong, specific, systemic, T cell-dependent anti-tumour immunity can result from the injection of C. parvum locally, intralesionally, or mixed with irradiated cells and given s.c. (Bomford, 1975; Scott, 1974b, 1975; Woodruff and Dunbar, 1975). This specific immunity is probably cell-mediated and directed at tumour associated transplant antigens (Bomford, 1975; Scott, 1975). Development of specific immunity seems to be partly dependent on the immune response to C. parvum itself when C. parvum is given locally or in admixture with irradiated cells (Scott, 1974b), but not when given intratumour (Scott, 1975). It is clear that C. parvum administration can, depending on circumstances,

initiate non-specific or specific anti-tumour responses.

Another consequence of the administration of C. parvum, and one which may have an important bearing on the in vivo development of activated macrophages and anti-tumour responses in man and animals, is the activation of both classical and alternate pathways of complement, (McBride, Weir, Kay et al., 1975c; Biran, Moake, Reed et al., 1976).

Recent studies on the distribution and persistence of C. parvum in the body have, to some extent, helped to explain some of the above observations. Thus biologically active strains of C. parvum persist in the body for much longer periods than inactive strains and C. parvum injected subcutaneously tends to remain at the inoculation site whereas C. parvum injected i.v. or i.p. is distributed to the lungs, spleen, liver, gut and bone marrow (Dimitrov et al., 1977; Sadler et al., 1977; Scott and Milas, 1977).

# PURPOSE AND SCOPE OF THE STUDY

PURPOSE AND SCOPE OF THE STUDY

The final sections of the foregoing introduction provide a brief outline of our knowledge to date of the effects of C. parvum administration in man and in animals, and of the possible mechanisms underlying its anti-tumour properties. At the commencement of this study, in the autumn of 1974, a great deal of this knowledge had already been obtained. For example, the importance of macrophages as anti-tumour effector cells could be deduced, as outlined above, from the RES stimulating capacity of C. parvum, the observation of anti-tumour effects in T cell-deprived and nude mice, the stimulation of bone marrow macrophage colony formation, and the cytotoxicity of peritoneal macrophages from C. parvum-treated T cell-normal and T cell-deprived mice. However, the precise mechanisms leading to activation of this macrophage anti-tumour effect were not known. It was as an attempt to bridge this gap in knowledge that this study evolved.

On the basis of published work in other systems, several possible pathways might have led to the production of cytotoxic macrophages in C. parvum-treated animals.

a) Macrophages could be activated directly by C. parvum. Direct activation *in vitro* had been observed using double-stranded RNA and endotoxin (Alexander and Evans, 1971) and by a fraction obtained from *Mycobacterium smegmatis* (Juy and Chedid, 1975). Circumstantial evidence of a direct effect of C. parvum on macrophages was already available from the work of Wolmark, Levine and Fisher (1974) showing a dramatic rise in the number

of bone marrow macrophage colony-forming units, peaking at 48 hours, following C. parvum administration. This early effect of C. parvum suggested a direct effect on macrophage precursors.

b) In view of the well known adjuvant effect of C. parvum (Howard et al., 1973) antibodies to C. parvum and/or tumour cells could play an important part in macrophage activation. Activation might then occur through the formation of immune complexes (Evans, 1975), or through activation of complement (see McBride et al., 1975c). Complement cleavage products were known to be chemotactic for macrophages and to be associated with various features of activated macrophages (see Snyderman and Mergenhagen, 1976).

c) The possibility had also to be considered that many of the C. parvum results were actually due to an antibody-dependent macrophage-mediated cytotoxicity as described, for example, by Dennert and Lennox (1973).

d) Macrophages might also be activated by sensitised lymphocytes or their products as described by several groups (Evans and Alexander, 1972a, b; Lohmann-Matthes et al., 1972; Pels and Den Otter, 1974; Hibbs, 1975a, b).

An investigation of three of these possibilities (a, b and d), using *in vitro* techniques, formed the initial objective of the project. The experimental design and the choice of materials and methods were dictated largely by previous, and by current, work on the anti-tumour effects of C. parvum carried out under the direction of Sir Michael Woodruff in the Department of Surgery, Edinburgh University.

Dr Abdul Ghaffar and myself had developed an _in vitro_ system utilising 96-well microculture plates to study the effects of lymphoid cells from _C. parvum_-treated mice on monolayers of methylcholanthrene (MC)-induced fibrosarcoma cells. Anti-tumour activity was assessed by measuring incorporation of $^{125}$IUDR into the tumour cells following their exposure to lymphoid cells. The reasonsfor this choice of method are detailed in Materials and Methods.

These _in vitro_ studies (Ghaffar _et al_., 1974, 1975) had already provided useful information on the mechanism of action of _C. parvum_. Thus, as in other reports (e.g. Olivotto and Bomford, 1974), the principal effector cell appeared to be the activated macrophage. Furthermore, the anti-tumour effects were mediated by intact macrophages and not by factors released into the culture medium. Attempts to probe the mechanism leading to the production of activated macrophages met with no success. Thus, macrophages could not be stimulated _in vitro_ by exposure to _C. parvum_, either alone, or in combination with lymph node cells from _C. parvum_-treated mice. This finding together with the observation that the production of _C. parvum_-activated macrophages was T cell-independent, indicated that the mechanism involved differed from the macrophage-arming system described by Evans and Alexander (1972a, b).

However, these negative findings had been obtained from only limited experimentation and a re-examination of this work eventually confirmed that macrophages could be activated _in vitro_ by lymphocytes from _C. parvum_-treated mice. An analysis of this mechanism, using methods then in current use such as T cell-deprivation of mice,

x-irradiation, gold salts, and antisera to T cells and macrophages, formed the bulk of the project reported in this thesis.

In parallel to this study, but not entirely divorced from it, was an investigation of the observation that *C. parvum*-activated macrophages quickly lost their cytotoxic potential when cultured *in vitro* but that this loss of activity could be delayed by the presence of *C. parvum*. The findings of this study are reported in Results, Part 2.

# MATERIALS AND METHODS

## MICE

Mice of the CBA/Ca strain were inbred in the Department of Surgery, Edinburgh University. Breeding pairs were originally obtained from the Laboratory Animal Centre, Carshalton, Surrey, England.

Only female mice, 9 to 12 weeks old were used. Experimental mice were housed in plastic boxes lined with wood shavings, 3 to 6 mice per box. Water and mouse cake (McGregor & Co. (Leith) Ltd., Edinburgh) were available to animals *ad libitum*. The mouse room had a 10 hour day and was maintained at an average temperature of 21°C with continual air exchange to ensure adequate ventilation.

## T CELL-DEPRIVED MICE (B MICE)

CBA/Ca mice were thymectomized at the age of 4 to 6 weeks. Ten days later these mice were given a potentially lethal dose (850 Rad) of whole body x-irradiation followed by an intravenous injection of $5 \times 10^6$ viable isogenic bone marrow cells. Mice were not used experimentally for at least 6 weeks following this procedure. The criteria for thymic deficiency in such mice (Woodruff, Dunbar and Ghaffar, 1973) were that they failed to mount known T cell-dependent immune reactions such as the production of antibody to sheep erythrocytes, and the rejection of H-2 incompatible skin allografts. All experimental B mice were examined when sacrificed to ensure that they had no thymic remnants.

## TISSUE CULTURE

### Growth Medium

RPMI-1640 medium buffered with 20mM HEPES* and supplemented with 10% heat-inactivated foetal calf serum (FCS), 2mM glutamine, 100 units $ml^{-1}$ penicillin, and 100µg $ml^{-1}$ streptomycin was used for tumour cell propagation and for the anti-tumour assays. The medium, glutamine and FCS were obtained from Gibco-Biocult, Glasgow, Scotland and the antibiotics in the form of Crystamycin from Glaxo Laboratories Ltd., Greenford, England. Details of the composition of RPMI-1640 are given in the appendix.

### Wash Medium

This medium, used for washing tumour cells, lymphoid cells and cell monolayers, was composed as for growth medium but with only 5% FCS.

### Phosphate-buffered saline (PBS)

Phosphate-buffered saline (pH 7.4), when used, was Dulbecco's formulation (see appendix) and was obtained from Oxoid, London.

### Tumour

The tumour studied was a fibrosarcoma, originally induced in the thigh of a female CBA mouse with a single intramuscular injec-

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*HEPES = N-2-hydroxyethylpiperazine-$N^1$-2-ethanesulphonic acid

tion of 0.5mg 3-methylcholanthrene suspended in 0.1 ml trioctanion (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The tumour, which is stored as single cell suspensions in liquid nitrogen, is now in its 18th transplantation generation. $1 \times 10^6$ of the stored cells are injected subcutaneously into the thigh of a CBA mouse to give one more transplantation passage before using the tumour in tissue culture.

The tumour, which has a TD50 of about 30 viable cells (Woodruff and Dunbar, 1974), is immunogenic in CBA mice, a single subcutaneous (s.c.) injection of irradiated (22,000 rad) cells completely protecting against challenge with $10^4$ viable tumour cells 14 days later (Woodruff and Dunbar, 1973).

## Preparation of tumour cell suspensions from solid tumour

Tumour cell suspensions were prepared by the following method based on that of Woodruff and Boak (1966) and originally adapted from Boyse (1960).

(1) 14-18 days following subcutaneous injection in the thigh of $1 \times 10^6$ stored tumour cells, when the tumour was about 14mm in diameter, the mouse was killed and the tumour was excised aseptically.

(2) The tumour was transferred immediately to a sterile glass universal container containing 10ml of a concentrated antibiotic solution ($10^3$ units $ml^{-1}$ penicillin, $10^3$ µg $ml^{-1}$ streptomycin, and $5 \times 10^3$ units $ml^{-1}$ mycostatin (Gibco-

Biocult), in RPMI-1640 medium). The use of antibiotics in such high concentration is an effective way of reducing possible microbial contamination to a minimum (Paul, 1970).

(3) After 10 minutes at room temperature in the antibiotic solution, the tumour was cut up into small pieces (2-4mm$^3$) with a pair of scalpels (No. 10 blades), discarding necrotic tissue in the process, and transferred to a universal container containing 2.4mg ml$^{-1}$ pronase (Calbiochem Ltd., Bishops Stortford, Herts., England) and 0.04mg ml$^{-1}$ deoxyribonuclease (DNA-ase; Sigma Chemical Co.) in Dulbecco's PBS. DNA-ase is important in the digestion system as it breaks down viscous nuclear protein which would otherwise cause clumping of cells and interfere with settlement.

(4) Digestion was allowed to continue with frequent vigorous shaking for 30 minutes in a 37$^{\circ}$C water bath. Large pieces of tumour were then allowed to settle and supernatant was drawn off by pipette and transferred to another universal for centrifugation (180g for 7 minutes). The tumour cells were washed a total of three times in PBS containing 0.04 mg ml$^{-1}$ DNA-ase. Cells were finally suspended in RPMI-1640 growth medium.

(5) Steps 3 and 4 were repeated until most of the tumour had been digested. This method normally yields 92-97% viable cells.

In vitro propagation of tumour cells

Tumour cells were grown in monolayer in glass tissue culture

bottles ($40cm^2$); $4 \times 10^6$ viable cells in 10ml RPMI growth medium per bottle for the initial passage, and $8 \times 10^5$ cells per bottle for subsequent passages. Cells were usually subcultured on every second day, the cell monolayers being dislodged by firmly tapping the glass bottle. Single cell suspensions were obtained by gently aspirating dislodged cells in a 5ml pipette.

Tumour cells were maintained in culture for 18-20 days before use in the assay system as it was found that cell adherence to plastic culture plates improved after this time. This period in culture also ensures the complete loss of any macrophages and lymphocytes present in the original tumour suspension. The cultured cells do not lose their capacity to produce a tumour when injected into mice.

The anti-tumour assays were carried out in 96-well microculture plates (Linbro, IS-FB-96, obtained from Flow Laboratories, Irvine, Scotland). Plates were incubated at 37°C in a humid atmosphere enriched with 5% $CO_2$. In one experiment 35mm diameter plastic Petri dishes (A/S Nunc, Denmark) were used in place of microculture plates.

It should be noted that the MC-induced tumour cell line used in this study has been screened for the presence of viruses and other microorganisms by a number of procedures including standard culture techniques, electron microscopy, immunofluorescence, RNA incorporation studies and reverse transcriptase assays. Tumour cells from both short (3 days) and long-term (8 months) culture lines have so far proved to be free of microbial contamination as judged by these tests (James et al., 1978). Such screening of tumour

cells is essential for immunological studies in view of the known influence of microorganisms and their products on immune responses (Kamo and Friedman, 1977). The 18-20 day culture of tumour cells before use in the anti-tumour assays would in itself reduce the possibility of one common type of tumour cell contaminant, lactate dehydrogenase elevating (LDH) virus, which has a poor survival under tissue culture conditions (Riley, 1968).

## EFFECTOR CELLS

### Spleen Cells

Untreated (control) and adjuvant-treated mice were killed by ether inhalation and their spleens excised aseptically. Spleens were gently disrupted in cold ($4^{o}C$) RPMI wash medium in loose-fitting, hand-operated glass homogenisers. After allowing large clumps of cells and splenic debris to settle for about 1 minute, the supernatant spleen cell suspensions were transferred to 10ml plastic centrifuge tubes and spun at 240g for 5 minutes. Spleen cell pellets were resuspended in 10ml fresh wash medium and respun, This washing procedure was repeated once more before resuspending the cells in cold RPMI growth medium, and making a nucleated cell count in a Neubauer haemocytometer.

Spleen cells were then incubated at $37^{o}C$ in large culture flasks (surface area $120cm^2$) for a total of 3 hours to remove adherent cells, particularly macrophages. Each flask contained a maximum of $5 \times 10^7$ cells in 30ml RPMI growth medium and, midway through the incubation, flasks were shaken to resuspend non-adherent cells and turned through

$180^{o}$ to ensure as complete a removal of glass-adherent cells as possible. Following incubation, non-adherent cells were transferred to centrifuge tubes, washed once, resuspended in growth medium and nucleated cells counted.

Lymph Node Cells

Mice treated by the intraperitoneal route had their inguinal, brachial and axillary nodes excised, and mice treated by the subcutaneous route had only those nodes local to the injection site removed. Nodes from untreated mice served as controls. Non glass-adherent lymph node cell suspensions were made according to the procedure described for spleen cells.

PREPARATION OF MACROPHAGE MONOLAYERS

Experiments on the in vitro activation of anti-tumour activity in normal macrophages

Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity of ether-killed normal (i.e. untreated) mice with two injected 2.5ml aliquots of RPMI-1640 medium containing per ml, 10 units preservative-free heparin (Evans Medical Ltd., Liverpool, England), 100 units penicillin, and 100 µg streptomycin. Washings were withdrawn by syringe after massaging the abdomen. $3\text{-}4 \times 10^{6}$ PEC were usually obtained in this way from each mouse.

Cells were washed three times in cold RPMI wash medium, a nucleated cell count made, and cells seeded in 96-well microculture plates to give $8 \times 10^{4}$ cells in 0.1 ml volumes per well. After $1\frac{1}{2}$

to 2 hours incubation at $37^{o}C$, wells were washed three times with 0.3ml volumes of wash medium to remove non-adherent cells. To aid this removal of unwanted cells, plates were shaken for 60-second periods on a Cooke Microtiter AM 69 Microshaker (Dynatech Labs. Ltd., Billingshurst, Sussex, England).

This method gives monolayers consisting predominantly of macrophages as determined by morphology, phagocytosis, adherence, and histochemical staining. Macrophages were enumerated using the following tests:

(1) Saline containing 0.1% neutral red added to adherent cell monolayers resulted in the rapid uptake of the dye to macrophages (Gesner and Howard, 1967).

(2) Phagocytic cells were identified by first incubating monolayers with C. parvum overnight. Monolayers were then washed thoroughly with PBS to remove extracellular bacteria, fixed in methanol, and stained with Gram's stain. Cells (stained red) which had phagocytosed bacteria (stained blue) were then easily enumerated.

(3) Differential counts were made of stained monolayers using (a) Leishman's stain and (b) Kaplow's method for myeloperoxidase (Kaplow, 1965).

On the basis of these tests it was found that the macrophage was the predominant monolayer cell, generally accounting for 92 - 97% of adherent cells. Other cells present in the monolayers were mostly lymphocytes with an occasional granulocyte ($<1\%$).

Experiments on the in vitro behaviour of peritoneal macrophages which had been stimulated in vivo by C. parvum

Macrophage monolayers were prepared as above from the peritoneal exudate of untreated mice and of mice which had been injected (usually 10 days before) intraperitoneally with 1.4mg C. parvum.

## ANTI-SERA

### Theta-1.2 (θC3H) Anti-Serum

The mouse theta (θ) alloantigen, first described by Reif and Allen (1963, 1964), has been found in brain, on epidermal cells (Scheid et al., 1972), fibroblasts (Stern, 1973), thymocytes and, to a lesser extent, in spleen and lymph nodes. The relatively low levels of theta in the peripheral lymphoid organs compared to the thymus, suggested a use for theta as a surface marker to distinguish thymus-derived lymphocytes (T cells) from thymus-independent lymphocytes (B cells)(Schlesinger and Yron, 1969; Raff, 1969). Theta antigen is the expression of one of two alleles: Thy-1.1 (θAKR) found in AKR and RF mice and Thy-1.2 (θC3H) found in most other inbred strains including CBA (Reif and Allen, 1966).

Anti-sera cytotoxic for theta-bearing lymphocytes were used in this study to remove the T cell population from spleen cells. Anti-Thy-1.2 serum was prepared by the following method adapted from Reif and Allen (1966).

A suspension of thymocytes was prepared by gently disrupting female CBA thymuses in a loosely fitting glass homogeniser. Cells were suspended in Dulbecco's PBS containing 0.05mg$^{-1}$ DNA-ase and washed

twice with this medium. 10 x $10^6$ thymocytes were then injected intraperitoneally into each AKR female mouse, the inoculation of thymocytes being repeated on days 2, 21, 30, 38 and 56. Mice were bled out on day 70 and the serum separated and heat inactivated (56°C for 30 minutes). Fifty per cent cytotoxic titres of the anti-serum to CBA spleen cells, and to thymocytes, were determined using the following assay:

(1) Doubling dilutions of anti-serum were prepared in plastic tubes using RPMI 1640 medium (without FCS) as diluent (0.1ml volumes).

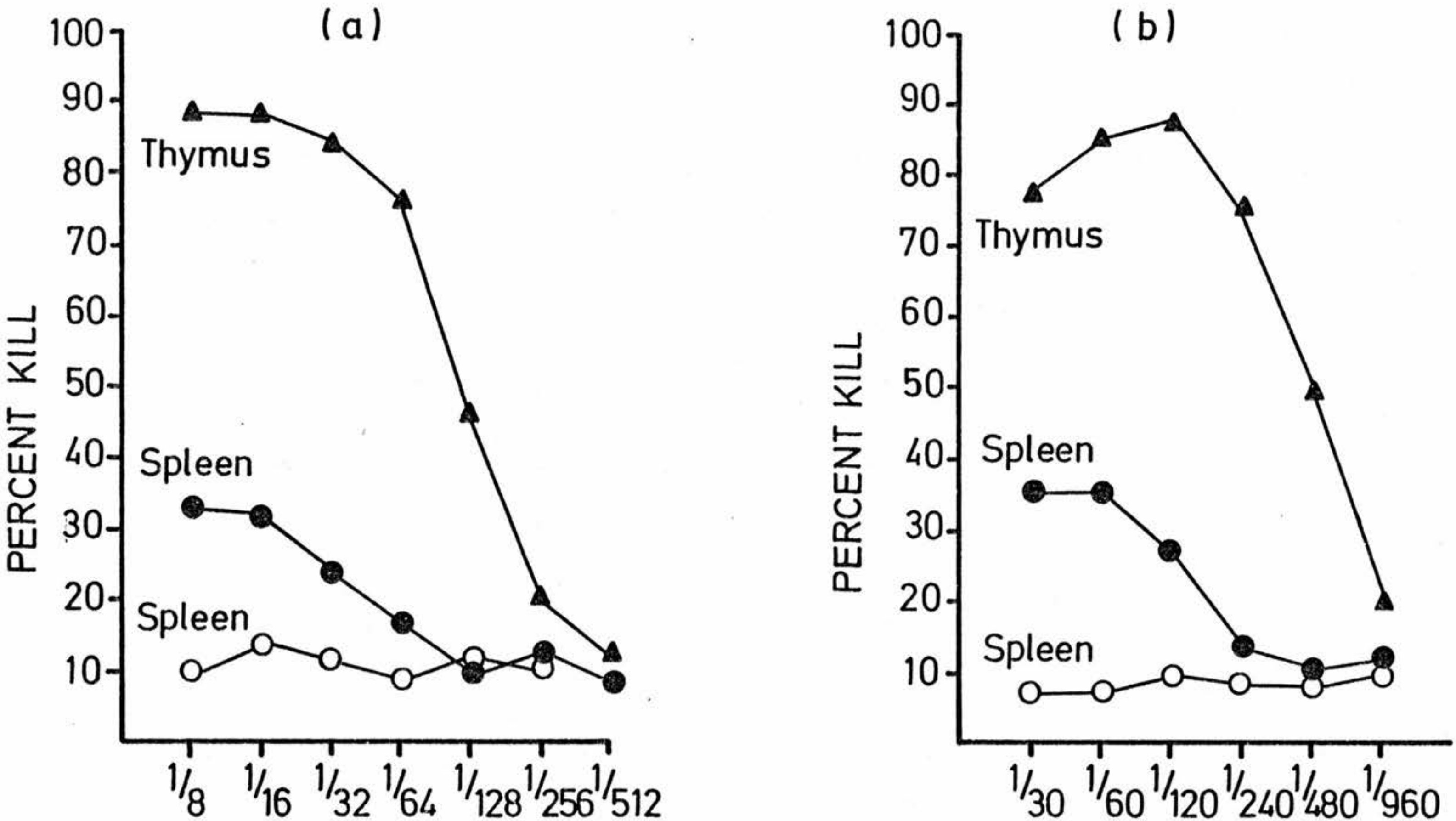
(2) 1 x $10^6$ spleen cells or thymocytes in 0.2ml were added to each tube and the tubes vortexed.

(3) Tubes were left at room temperature (20°C) for 15 minutes.

(4) 0.1 ml of 1:4 dilution of agarose-absorbed guinea pig complement (see below for details) were added to each tube which was then vortexed. A tube without anti-serum was included to act as complement control. Tubes were incubated for 1 hour in a 37°C water bath.

(5) 0.1ml trypan blue (see appendix) were added to each tube which was vortexed and left at 37°C for 5 minutes.

(6) 0.1ml formalin were added to each tube. The number of dead cells (those which had taken up trypan blue) was expressed as a percentage of the total number of cells per tube and plotted with respect to serum dilution. Figure 1(a) illustrates the results using anti-Θ serum raised by the method described above and figure 1(b) the results using a commercial anti-Θ serum (Searle and Co. Ltd., High Wycombe, England).




<u>FIGURES 1(a)-1(b)</u> <u>CYTOTOXICITY OF ANTI-Θ SERA FOR NORMAL SPLEEN CELLS ( ● ) AND THYMOCYTES ( ▲ )</u>

Open circles (O) represent the toxicity of normal mouse serum for spleen cells. Figure 1(b) illustrates results using a commercial anti-Θ serum.

In order to kill theta-bearing cells in spleen populations it is necessary to use the anti-serum at a concentration which is on a cytotoxic plateau (Raff, 1971). Thus from Fig.1a, the anti-theta serum was used at a final concentration of 1:8. Spleen cells depleted of glass-adherent cells were treated with anti-serum and complement as above, but scaling up the volumes and numbers of cells. Following the treatment the spleen cells were washed twice, resuspended in growth medium, and their concentration adjusted to give $4 \times 10^6$ cells $ml^{-1}$ ready for use in the activation assay. Control groups of spleen cells treated only with complement were also included in the test.

Guinea Pig Complement

Normal guinea pig serum is generally toxic for mouse thymus (e.g. Schlesinger, 1965) and accordingly has to be absorbed with murine tissue before use as a complement source. An alternative to the use of mouse cells for absorption was described by Cohen and Schlesinger (1970) in which cytotoxicity to murine thymus cells can be effectively absorbed with a carbohydrate source such as agarose.

Guinea pigs were anaesthetised (Nembutal) and bled by cardiac puncture. Serum was diluted 1:2 with RPMI-1640 (without FCS) and 80mg agarose (Agarose A37, L'Industrie Biologique Francais, Genne-villiers, France) for every original 1ml of serum added. Serum plus agarose was placed in a refrigerator. The container was inverted six times and this was repeated at 5-10 minute intervals for 1 hour. The serum was then centrifuged in a cold room ($4^oC$) at 1500g and the supernatant serum transferred to a glass universal container and stored at $-20^oC$ until use.

Anti-macrophage Serum

Rabbit anti-mouse macrophage serum was prepared using the method of Unanue (1968). Peritoneal exudate cells from CBA female mice were cultured in 35mm plastic Petri dishes (approx. $15 \times 10^6$ cells/dish) for a period of three days at 37°C in a humid atmosphere enriched with 5% $CO_2$. The monolayers were washed (two medium changes) at 30 minutes, 24 hours and 48 hours. This culture method effectively removes most mast cells, polymorphonuclear leucocytes and lymphocytes (See Hirsch et al., 1969) leaving a population which is more than 95% macrophage. After three days in culture the cell monolayers were washed twice with medium, detached with a "rubber policeman" and washed twice in Dulbecco's PBS. New Zealand White rabbits were immunized and bled according to the following protocol:

Day 0 $12 \times 10^6$ CBA macrophages in complete Freund's adjuvant injected into each rabbit, the injections being distributed between four sites: hind footpads (0.15ml each) and forearms (0.3ml each).

Day 28 $6 \times 10^6$ CBA macrophages in Dulbecco's PBS (0.6ml) intravenously (ear).

Day 35 50 ml bled and $6 \times 10^6$ macrophages in Dulbecco's PBS injected intravenously (ear).

Day 42 Rabbits bled out.

The serum was heated at 56°C for 30 minutes, sterilised by Millipore (0.2μm) filtration and stored at -20°C. Each 1ml of anti-

serum was absorbed with 4 x $10^7$ CBA thymocytes for 30 minutes at $4^{\circ}C$. Cells were pelleted by centrifuging at 240g for 5 minutes and the serum transferred to another tube containing a second batch of 4 x $10^7$ CBA thymocytes. This tube was left overnight at $4^{\circ}C$. Fifty per cent cytotoxic titres to thymocytes and cultured peritoneal macrophages were carried out on the anti-macrophage serum using the method described above for anti-theta serum, using guinea pig serum as complement source. The batch of anti-macrophage serum used in this study had 50% cytotoxic titres of 1:512 against macrophages and 1:4 against thymocytes.

## ADJUVANTS

Formalin-killed suspensions of *Corynebacterium parvum* strain CN6134, containing 7 mg dry weight of organisms per ml were obtained as a gift from the Wellcome Research Laboratories, Beckenham, Kent.

In one *in vitro* experiment, two other corynebacteria, *C. parvum* NCTC 10390 and *Propionibacterium freudenreichii* NCTC 10470, were compared with *C. parvum* CN6134. Formalin-killed suspensions of these bacteria, prepared as previously described (McBride, Dawes, Dunbar, Ghaffar and Woodruff, 1975a), were kindly provided by Dr. W.H. McBride of the Department of Bacteriology, University of Edinburgh.

It is recognised that "*C. parvum*" is a general designation for a heterogeneous group of anaerobic bacteria that have proved difficult to classify. These bacteria can be divided into four taxonomic groups (I - IV) on the basis of serology, cell wall composition, and DNA homology (Johnson and Cummins, 1972; Cummins and Johnson, 1974). As a result of such studies it has been proposed that the "*C. parvum*"

bacteria be in fact classified under *Propionibacterium* (Douglas and Gunter, 1946; Johnson and Cummins, 1972; Cummins and Johnson, 1974). However, as there remains controversy over the true taxonomic position of these bacteria (see McBride *et al.*, 1975a), the classical name *C. parvum* has been retained in this study. Adopting the taxonomic grouping of Johnson and Cummins (1972), the *C. parvum* strains 6134 and 10390 used in this study have been placed in groups I and II respectively (McBride *et al.*, 1975a).

Freeze-dried BCG (Bacille-Calmette-Guérin) vaccine, containing, when reconstituted, approximately $9 \times 10^6$ organisms per ml, was purchased from Glaxo Laboratories, Greenford, Middlesex, and a *Bordetella pertussis* suspension containing $4 \times 10^{10}$ killed organisms per ml was purchased from Wellcome Reagents Ltd., Beckenham, Kent.

The dose used per mouse of a bacterial adjuvant was 0.2ml of the original suspension and in most experiments this was administered intraperitoneally. In experiments employing the subcutaneous route, the dose was divided equally between two sites, the right foreleg and the right hindleg. Intravenous injections were given via a large tail vein, the tail having first been warmed in warm water.

When used *in vitro* the bacteria first had to be thoroughly washed to remove all traces of preserving chemicals. This involved washing 0.1ml aliquots of the bacterial suspensions six times in 10ml volumes of PBS. Washed bacteria were resuspended in RPMI growth medium to give a stock containing one tenth of the concentration of the original suspension. The stock suspensions were further diluted 1:100 before use in an experiment. Thus, *C. parvum* CN6134 was finally

diluted to give 7µg $ml^{-1}$ and 0.1ml of this was added to each well of a microculture plate. Washed preparations of C. parvum strain 10390 and P. freudenreichii strain 10470 were standardised to the C. parvum strain 6134 preparation by optical density.

Freund's complete and incomplete adjuvants, obtained from Difco Laboratories, Detroit, Michigan, U.S.A., were injected i.p. at a dose of 0.1ml per mouse. These adjuvants were not tested in vitro.

GOLD SALT TREATMENT

The effect of sodium aurothiomalate ('Myocrisin', containing 45% metallic gold; May and Baker Ltd., Dagenham, Essex) on the in vitro stimulation of anti-tumour cytotoxicity in normal macrophages, was studied in vitro and in vivo.

In the in vivo experiments the following regimen of injections was followed:

Group 1 1 mg gold salt in 0.2ml saline i.p. on days -8, -6, -4 and -1. 1.4mg C. parvum i.p. on day -7.

Group 2 1 mg gold salt in 0.2ml saline i.p. on days -8, -6, -4 and -1.

Group 3 1.4mg C. parvum i.p. on day -7.

Group 4 No treatment.

Mice were killed on day 0 and their spleens harvested.

This schedule was determined by the results of a previous investigation (McBride, Tuach and Marmion, 1975) using the same

fibrosarcoma used in this study, which had shown that, in order to abrogate the inflammatory response to C. parvum and its in vivo anti-tumour action, it was necessary to administer the gold salt as a series of injections starting before the C. parvum injection.

For the in vitro experiments various concentrations of sodium aurothiomalate (10, 20 and 50μg) were added to wells, either during macrophage adherence, or during the macrophage activation process.

The in vitro effect of gold salt on the maintenance of anti-tumour cytotoxicity by C. parvum in macrophages from C. parvum-treated mice was also investigated.

## X-IRRADIATION

Mice were irradiated in perspex boxes at a dose of 60 rad per minute using a 230 kV Westinghouse X-ray machine, operating at 15ma with a half-value layer of 1.2mm Cu under conditions of maximum back scatter. Spleen and peritoneal exudate cells in 60mm diameter plastic Petri dishes were irradiated at the same dose rate.

## SEPARATION OF SPLEEN T AND B LYMPHOCYTES ON NYLON WOOL COLUMNS

Spleen cell separation was carried out using a nylon wool column filtration method, based on that described by Julius, Simpson and Herzenberg (1973). This, and other studies (Eisen, Wedner and Parker, 1972; Greaves and Brown, 1974; Handwerger and Schwartz, 1974) have shown that the effluent cell population from the column is enriched with T lymphocytes and the nylon adherent population with B lymphocytes (Handwerger and Schwartz, 1974).

About 10g of the nylon wool (LP-1 Leuko-Pak, Leukocyte filter, Fenwall Laboratories, Morton Grove, Illinois, U.S.A.) was soaked in 250ml distilled, deionised water and kept at 37°C for three days, changing the water each day. This washing improves post separation viability of cells in culture (Julius et al., 1973; Greaves and Brown, 1974).

The washed wool was dried and 600 mg quantities were packed to the 5ml mark in the barrels of 5ml plastic syringes. The packed syringes were wrapped in Kraft paper and autoclaved. Sterile plastic 2-way taps were fitted to the syringe barrels and the nylon wool columns rinsed with 25ml warm (37°C) RPMI wash medium, drained of excess medium, sealed, and equilibrated at 37°C for 1 hour. $1 \times 10^8$ non-adherent, glass-incubated spleen cells (vide supra) in 2ml wash medium were loaded onto the column and washed into the wool with 1ml of warm wash medium. Columns, which were maintained in a vertical position throughout the separation procedures, were incubated for 1 hour at 37°C.

Non-adherent cells were then eluted slowly be adding 15ml warm (37°C) wash medium drop by drop to the top of the column. Effluent cells were centrifuged at 240g for 7 minutes, resuspended in 2ml medium and loaded onto a fresh column. This was incubated for 30 minutes at 37°C and non-adherent cells recovered by washing the column slowly, as above.

Immunoglobulin-bearing cells which had been retained in the first and second columns were recovered by eluting with 25ml cold (4°C) wash medium and by simultaneous agitation of the nylon wool.

The separated cell populations were centrifuged, resuspended in

RPMI growth medium, and viable, nucleated cells counted in a haemocytometer. The relative numbers of B lymphocytes in the two separated populations and in the original untreated spleen population, were assessed by Mr T. Varley of the Department of Surgery, University of Edinburgh, using an indirect immunofluorescence staining method for cells carrying surface immunoglobulin light chains. Briefly, the method, which has been fully described elsewhere (McCormick, Nelson, Tunstall and James, 1973) involved incubating cells for 30 minutes at room temperature (20°C) with rabbit anti-mouse light chain serum followed by washing and 30 minutes incubation with fluorescein-conjugated goat anti-rabbit serum. Stained cells were then washed, deposited on microscope slides in a Shandon cytocentrifuge, and then air dried and mounted in barbitone (50% glycerol) buffer (pH 8.6). Slides were examined alternately with incident blue violet illumination and dark ground illumination.

## CHOICE OF IN VITRO ASSAY

A number of methods utilizing monolayers of target cells in tissue culture have now been developed to assess antibody-mediated and cell-mediated immunity to animal and human tumours. These assays measure either inhibition of growth of target cells (cytostasis) or actual killing or injury of target cells (cytotoxicity). In the latter assay, detachment of cells from the substrate is generally taken as a correlate of cytotoxicity, detached cells usually being seen to be dead or injured (Roseneau and Moon, 1961; Biberfield et al., 1968; Ax et al., 1968 and Oldham et al., 1973). However, all assays, cytotoxic or cytostatic, have in common an assessment of numbers of

cells remaining at the end of a test. The early work in this field involved visual counting of cells (Takasugi and Klein, 1970; Hellström et al., 1971), cell colonies (Hellström, 1967) or of cell nuclei (Rosenau and Moon, 1961; Wilson, 1963; Ulrich and Keiler, 1969). Such methods were tedious and lacked objectivity, it sometimes being difficult to distinguish morphologically between target cells and adherent lymphoid cells or to count accurately cells which had clumped in culture. These difficulties can be avoided by using an electronic counting system (Takasugi et al., 1973) or by labelling of target cells with radio-isotopes. Sodium ($^{51}$Cr)-chromate-labelled cells have been used in assays for antibody-induced lysis (Sanderson, 1964; Wigzell, 1965) and cell-mediated lysis (Holm and Perlmann, 1967; Brunner et al., 1968). The chromate is reduced on binding to cellular constituents and thus, during lysis of target cells, the 80-95% $^{51}$Cr released is in a non-reutilizable form (Bunting et al., 1963; Holm and Perlmann, 1967). Unfortunately the usefulness of the technique is marred by the relatively high spontaneous release of $^{51}$Cr, restricting assays to short incubation periods, generally between 3 and 10 hours. In assays requiring incubation periods in excess of 24 hours several methods have been developed which use nuclear DNA labels: $^{14}$C-Thymidine (Vainio et al., 1964); $^{3}$H-Thymidine (Jagarlamoody et al., 1971) and 5-($^{125}$I) Iodo-2-deoxyuridine ($^{125}$IUDR; Cohen et al., 1971). IUDR is a structural analogue of thymidine and as such can be incorporated into the DNA of dividing cells. An RNA label, $^{3}$H-Uridine, has also been used (Hashimoto and Sudo, 1971) but has the disadvantage that it is released spontaneously and can be reutilized. Reutilization is also a

problem with the DNA labels $^{14}C$-thymidine and $^{3}H$-thymidine but is not apparently a significant factor in assays using $^{125}$IUDR (Cohen et al., 1971; le Mevel et al., 1973 and Seeger et al., 1974). $^{125}$IUDR has a further advantage over $^{14}C$- and $^{3}H$- labels in that being a gamma-emitter, labelled cells do not require special preparation prior to scintillation counting.

Because of these advantages it was decided to use $^{125}$IUDR labelling in this study. It remained to choose between labelling the target cells (fibrosarcoma tumour cells) before or after their exposure to the effector cells (adherent peritoneal exudate cells in this study). The first procedure (pre-labelling) provides a measure of injury or death of target cells but the second fails to distinguish between such effects and mere growth inhibition (cytostasis) of targets. Since cytostasis may be just as important an anti-tumour mechanism as cytotoxicity and since the precise nature of C. parvum's anti-tumour effects were not known, it was decided to use the post-labelling technique. This procedure had the added advantage that the tumour cells are exposed to $^{125}$IUDR for a minimum period of time, thus reducing its reported chemical and radiation toxicity (Morris and Cramer, 1968; Hofer and Hughes, 1971; Porteous, 1971; le Mevel et al., 1973). (See also section below on incorporation kinetics and toxicity of $^{125}$IUDR).

## METHOD OF QUANTITATING TUMOUR CELLS IN MONOLAYERS USING $^{125}$I-IODODEOXYURIDINE

$^{125}$I-Iododeoxyuridine obtained from the Radiochemical Centre, Amersham, England was diluted with RPMI-1640 medium containing 100

unit $ml^{-1}$ penicillin and 100μg $ml^{-1}$ streptomycin to give a stock solution with radioactive concentration of 50μCi $ml^{-1}$ and this was stored at $4^{o}C$ until use.

Quantitation of $^{125}$IUDR-labelled tumour cells remaining in a monolayer at the end of an experiment was carried out using the following procedure which was standard for the majority of experiments in this study.

Cell monolayers in wells of microculture plates (Linbro, IS-FB-96) were gently washed twice with 0.2ml volumes of RPMI-1640 wash medium (vide supra) to remove unincorporated $^{125}$IUDR and non-adherent cells. Medium was withdrawn from wells using a plastic 10ml syringe and 19 gauge needle from which the point had been cut off. Care was taken to ensure as little disruption of the cell sheet as possible, and, to aid this, wells were never completely emptied, a film of medium (approx. 0.02-0.03ml/well) covering the cells at all times. Plates were then left to dry thoroughly at $37^{o}C$ and sprayed with a plastic aerosol preparation such as Trycolac MK IV (Aerosol Marketing and Chemical Co. Ltd., London, England) to fix cells to the wells. Individual wells were then cut out with a hot wire and the radioactivity counted using a LKB-Wallac 1280 Ultrogamma scintillation spectrometer (LKB Instruments Ltd., S. Croydon, Surrey, England).

## INCORPORATION KINETICS AND TOXICITY OF $^{125}$I-IODODEOXYURIDINE

Experiments were conducted to determine the best tumour cell density, $^{125}$IUDR concentration, and labelling time for the assay.

Kinetics /

Kinetics

Various concentrations of tumour cells were seeded in 0.1ml volumes in wells of microculture plates and incubated for 18 hours at 37°C. 0.1ml volumes of $^{125}$IUDR were then added to the wells and the plates incubated for varying periods up to 48 hours before washing the wells and assessing isotope incorporation as described in the preceding section. The variation of incorporated $^{125}$IUDR with increasing labelling time for each of the four $^{125}$IUDR concentrations used is given in figures2(a) - 2(d). Each observation is the geometric mean of the counts from four replicate wells.

Several points emerge from this experiment:

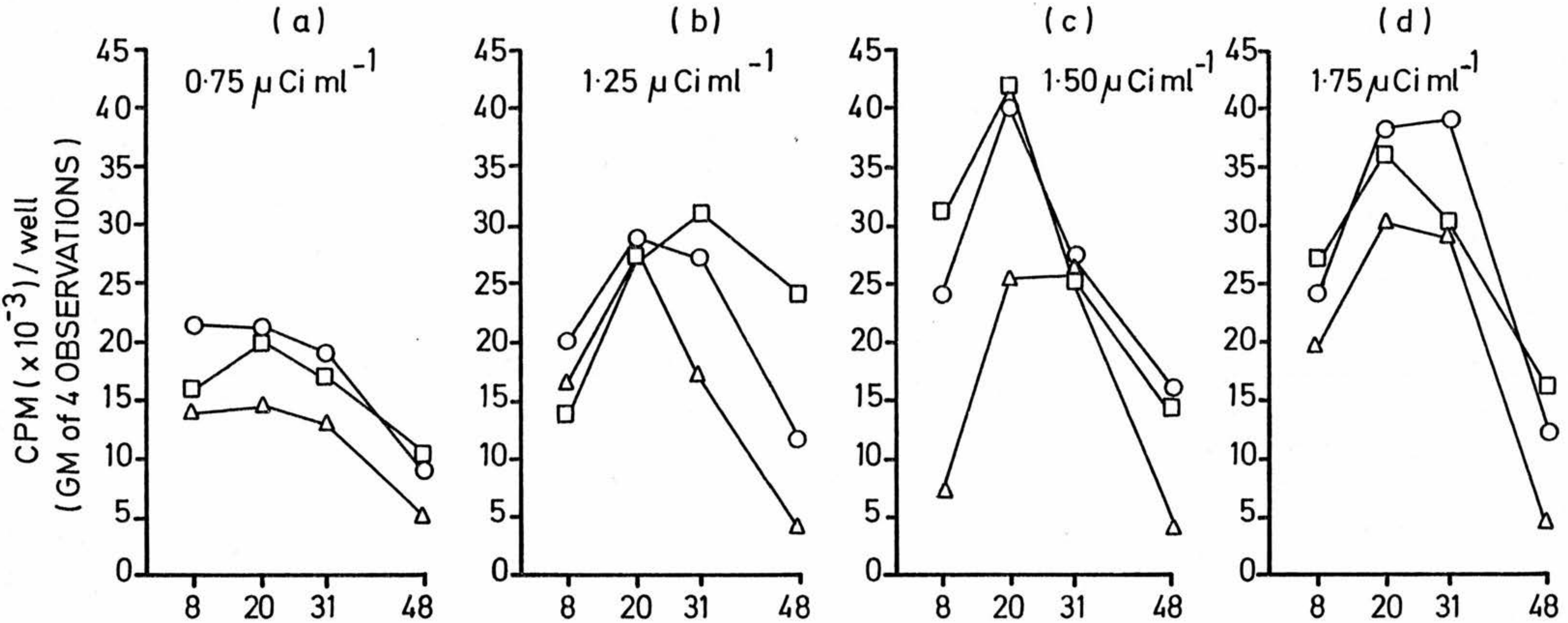
(1) As expected, the radioactive count generally increases with increasing cell density and increasing $^{125}$IUDR concentration. This is more apparent from figures 3(a) - 3(c) which show the variation in counts with increasing concentration of isotope, after 20 hours incubation.

(2) Incorporation reaches a maximum after 20-31 hours incubation and thereafter decreases.

(3) The low counts at 48 hours suggest that the label was starting to prove toxic for the cells.

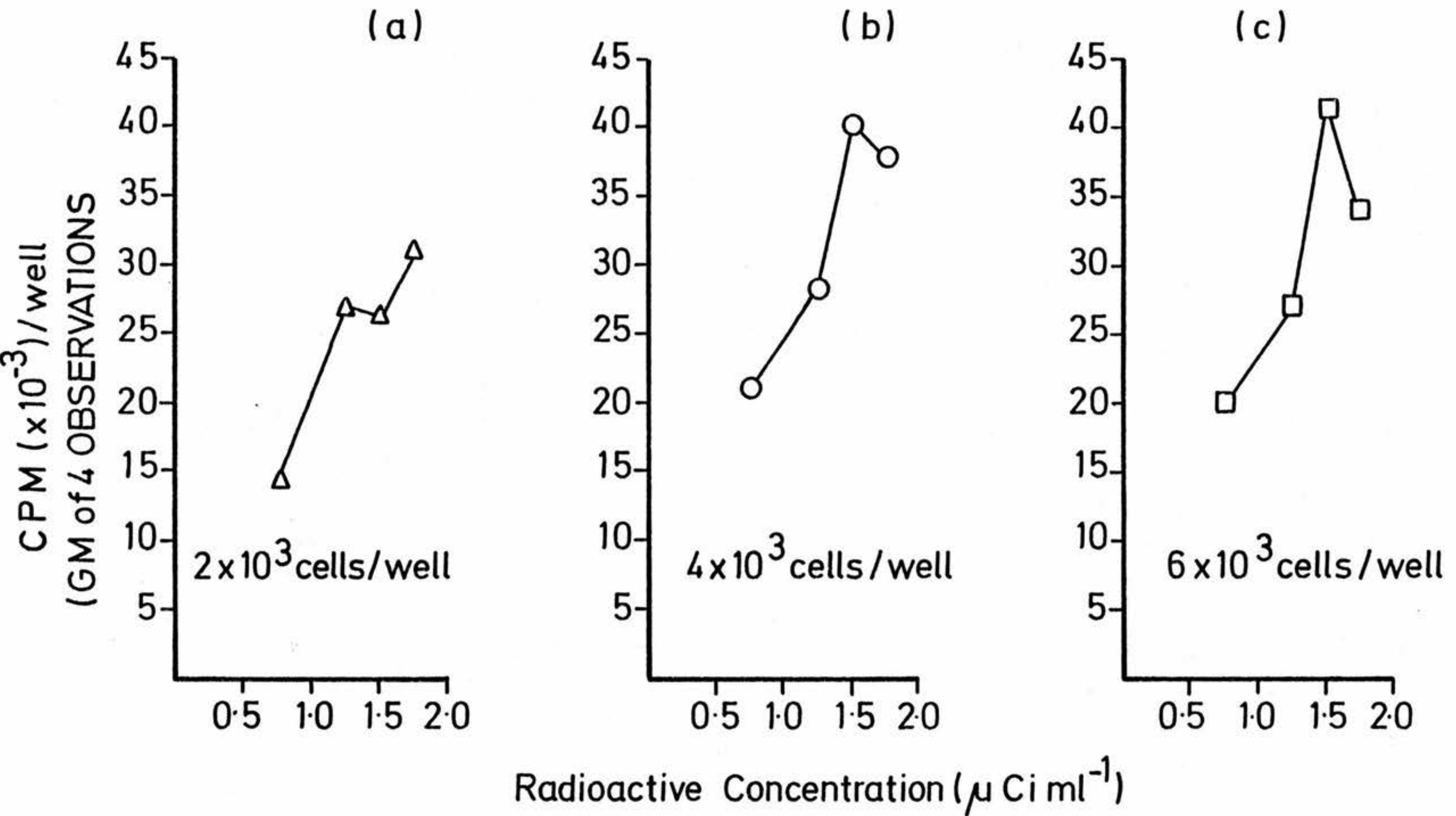
Toxicity

The toxicity of $^{125}$IUDR for MC fibrosarcoma cells was tested in a further experiment in which 4 x $10^3$ cells per well were seeded in a microculture plate and incubated with 1.25µCi $ml^{-1}$ $^{125}$IUDR for a




FIGURES 2(a)-2(d) EFFECTS OF CELL DENSITY, ISOTOPE CONCENTRATION, AND LABELLING TIME ON INCORPORATION OF $^{125}$IUDR IN MC FIBROSARCOMA CELLS.

Tumour cells were seeded in wells at various densities, $2x10^3$ (△), $4x10^3$ (○), and $6x10^3$ (□), and incubated for 18 hours. $^{125}$IUDR at various concentrations was then added to wells and the plates incubated for periods up to 48 hours. Final concentrations of $^{125}$IUDR in wells was 0.75, 1.25, 1.50 and 1.75 μCi $ml^{-1}$.




FIGURES 3(a) - 3(c) EFFECT OF INCREASING THE CONCENTRATION OF $^{125}$IUDR ON ITS UPTAKE BY FIBROSARCOMA CELLS DURING A 20 HOUR PERIOD

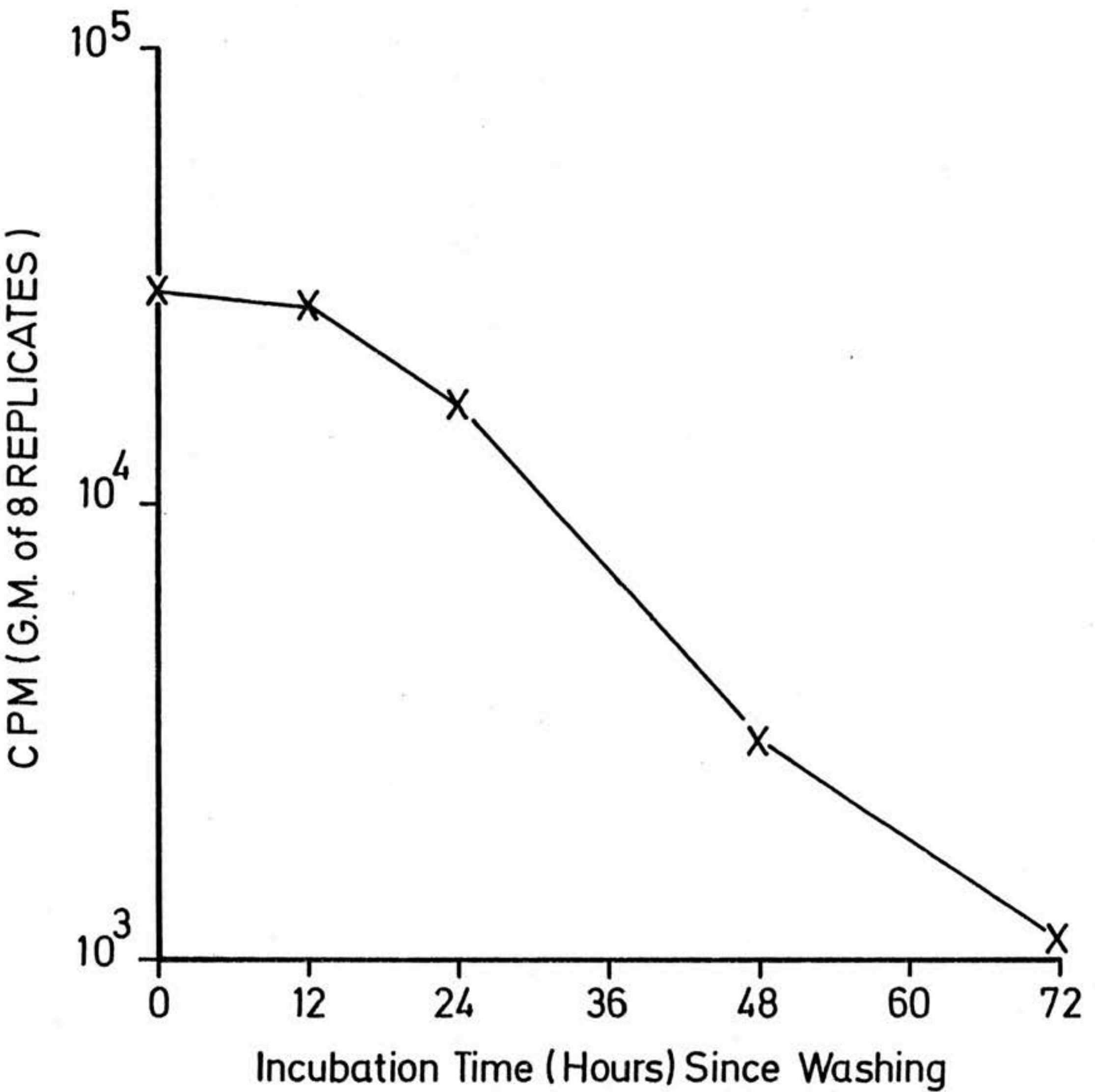



FIGURE 4 LOSS OF RADIOACTIVITY FROM $^{125}$IUDR-LABELLED FIBROSARCOMA MONOLAYERS DURING CULTURE.
Tumour cells labelled for 18 hours, washed and incubated for varying periods

period of 18 hours. The cell monolayers were then washed 3 times to remove unincorporated label and the plate incubated for various periods up to 72 hours. At the end of each period, adherent cells were harvested with 0.25% trypsin, washed once, pelleted and radioactivity assessed in the gamma counter. Loss of radioactivity from the monolayers with incubation is shown in Figure 4. Cells have either released isotope, or become detached from the monolayers.

From these, and other similar experiments not shown, it was decided to label tumour cells in the anti-tumour assay with $^{125}$IUDR at 1.25µCi $ml^{-1}$ over a period of about 20 hours. This isotope concentration was chosen because it gave fairly high counts and because any gain in incorporation levels obtained with the 1.75µCi $ml^{-1}$ concentration was outweighed by increased costs and, again, the desire to reduce any possible toxic effects. From Figure 3 it can be seen that at 20 hours and with a $^{125}$IUDR concentration of 1.25µCi $ml^{-1}$, original cell density has little effect on incorporation. Therefore, it was arbitrarily decided to use $4 \times 10^3$ tumour cells per well in future experiments.

<u>IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MORMAL MACROPHAGES</u>

For the majority of experiments, <u>in vitro</u> activation of macrophages was achieved by incubating macrophage monolayers together with <u>C. parvum</u> and spleen cells from <u>C. parvum</u>-treated mice. The following protocol, evolved from a series of pilot experiments, proved to be the most effective in producing activation of anti-tumour cytotoxicity.

$4 \times 10^5$ non-adherent spleen cells, obtained from mice injected

i.p. 6 to 12 days before with 1.4mg C. parvum (6134), were added to macrophage monolayers in wells of microculture plates, together with 0.7µg washed C. parvum (6134). The volume of growth medium in each well was maintained at 0.2ml throughout the experiment.

Plates were incubated overnight at 37°C in a humid atmosphere containing 5% $CO_2$. After 20 hours wells were washed three times with 0.3ml volumes of wash medium to remove spleen cells and unphagocytosed C. parvum. This was aided by placing the plates on a Cooke AM 69 microshaker between washes. $4 \times 10^3$ cultured tumour cells in 0.2ml growth medium were then added to each well and the plates incubated. 24 hours later 0.1ml was discarded from each well and replaced with 0.1ml growth medium containing 0.25µCi $^{125}$IUDR. Following a final incubation of 20 hours, wells were washed and $^{125}$IUDR incorporation assessed as described above. Generally, the cytotoxicity obtained using spleen cells from C. parvum-treated mice was compared with that using normal spleen cells.

Each plate in an experiment carried additional controls which assessed the cytotoxicity of the various activation components in alternative combinations and individually: Spleen cells ± C. parvum; macrophages ± C. parvum; C. parvum; spleen cells + macrophages. The spleen cell controls were necessary since the washing process always left a small (<0.1%) persistant population of adhering cells in the wells.

## EXPERIMENTS ON THE IN VITRO BEHAVIOUR OF MACROPHAGES FROM C. PARVUM TREATED MICE

Macrophage monolayers were prepared as described above using

peritoneal exudate from normal and *C. parvum*-treated mice. After various incubation periods 0.7µg *C. parvum* 6134 (or its equivalent in other bacterial adjuvants) was added to each well. Control wells received growth medium only. Plates were incubated for a further 24 hours before washing and shaking to remove bacteria. $4 \times 10^3$ tumour cells were added to each well, and cytotoxicity was assessed as before. In some experiments cytotoxicity was measured in the continued presence of *C. parvum*.

## PRESENTATION OF RESULTS AND STATISTICAL ANALYSIS

The geometric mean (GM) was calculated of the counts per minute from each experimental group of wells and recorded along with one standard error from the mean. The number of replicate wells in an experimental group was never less than three, and in most experiments was five. Mice were in groups of three to six.

The significance of data was assessed by the standard two tail Student's 't' test. P values greater than 0.05 were considered to be not significant. All statistical manipulations were performed using a Wang (Model 154) programmable electronic calculator (Wang Laboratories Inc., Tewksbury, Massachusetts, U.S.A.) and Geigy scientific tables (Documenta Geigy, 7th edition: published by J.R. Geigy S.A., Basle, Switzerland).

Some results have been expressed as a cytotoxic index (CI) calculated using the following formula:

$$CI = 1 - \left[\frac{\text{GM OF COUNTS FROM TEST GROUP}}{\text{GM OF COUNTS FROM CONTROL GROUP}}\right] \times 100$$

It should be noted that the term cytotoxic has been used throughout

this thesis in a general sense to cover possible cytostatic, cytocidal and cytolytic effects since the $^{125}$IUDR labelling technique fails to distinguish between such effects.

In experiments studying the maintenance of cytotoxicity in cultured macrophages by *C. parvum* some results have been expressed as a cytotoxic stimulation index (CSI):

$$\text{CSI} = 1 - \left[\frac{\text{GM OF COUNTS FROM WELLS WITH C. PARVUM}}{\text{GM OF COUNTS FROM WELLS WITHOUT C. PARVUM}}\right] \times 100$$

The CSI provides a measure of the extent to which the presence of *C. parvum* sustains the anti-tumour activity of cultured macrophages.

# R E S U L T S   P A R T   1

## *IN VITRO* ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN NORMAL MACROPHAGES

As described in the introduction, the purpose of this study was to attempt to elucidate the mechanism, or mechanisms by which C. parvum exerts its anti-tumour activity. Previous in vitro work (Ghaffar et al., 1974, 1975; Olivotto and Bomford, 1974) has made some progress towards a solution of this problem by demonstrating that the macrophage was the principal cytotoxic cell in peritoneal exudate from C. parvum-treated mice. The following in vitro experiments investigated possible ways in which this anti-tumour activity in macrophages might be brought about by C. parvum. The simplest model would require exposure of macrophages or macrophage precursors to C. parvum which might then lead to anti-tumour activation directly. Other mechanisms would involve T and/ or B lymphocytes, possibly operating via released factors or immunoglobulin specific for C. parvum antigens. It should be noted that the term activation used in this thesis simply implies stimulation of the capacity of macrophages to exhibit a cytotoxic reaction to the target fibrosarcoma cells. The term cytotoxic has been used in a general sense to include cytolytic, cytocidal and cytostatic effects since the post-labelling assay system used fails to distinguish between killing of target cells or mere inhibition of cell division.

## 1. ATTEMPTS TO STIMULATE MACROPHAGES IN VITRO DIRECTLY BY EXPOSURE TO C. PARVUM

A simple mechanism for activation seemed a reasonable supposition particularly since Alexander and Evans (1971) had reported on the anti-tumour behaviour of macrophages exposed in vitro to small amounts of double stranded RNA or endotoxin. Similarly, Juy and

Chedid, (1975) were able to obtain growth inhibition of tumour cells by macrophages previously exposed to a fraction obtained from *Mycobacterium smegmatis*.

Accordingly, experiments were conducted in which monolayers of peritoneal macrophages were incubated in the presence of various concentrations of a washed preparation of *C. parvum* 6134 for various time intervals up to 10 days. Tumour cells were then added to the monolayers and anti-tumour activity assessed by $^{125}$IUDR incorporation 48 hours later. None of these experiments (table 1) gave rise to macrophages exhibiting the degree of cytotoxicity known to occur in the peritoneal macrophages of *C. parvum*-treated mice (see table 20). Nevertheless slight activation was observed in certain groups, particularly those using greater amounts of *C. parvum*, but otherwise there was no consistent pattern.

Experiments were also conducted in which adherent peritoneal cells were incubated simultaneously with *C. parvum* and serum from *C. parvum*-treated mice. Sera were tested from mice which had received 1.4mg *C. parvum* intraperitoneally 4, 10 or 24 days previously. The rationale of this experiment was that formation of antigen/antibody complexes might activate macrophages (see Introduction, section 5.3). However, this approach also failed to induce anti-tumour activity (data not shown).

However, these results do not rule out a case for direct activation of macrophages *in vivo*. It is possible that macrophages which have ingested *C. parvum* may play a role in stimulating the maturation and release of promonocytes from bone marrow and that the resultant mature monocytes are the anti-tumour effector cells *in vivo* or

TABLE 1 EFFECT OF THE PRESENCE OF C. PARVUM DURING INCUBATION ON THE IN VITRO CYTOTOXICITY OF NORMAL PERITONEAL MACROPHAGES

| Incubation Pre-Tumour | Tumour Cells Alone | Amount of *C. parvum* added per well | | | | | |
|---|---|---|---|---|---|---|---|
| | | None | 3.5µg | 7.0µg | 14µg | 28µg | 56µg |
| 1 Day | 27,771 | 18,021 | 18,675 | 17,014 | 18,369 | 16,175 | 16,851 |
| 3 Days | 31,654 | 22,450 | 23,792 | 21,752 | 21,604 | 20,074* | 19,505* |
| 5 Days | 42,511 | 34,292 | 37,056 | 34,277 | 29,780* | 29,171* | 28,520* |
| 7 Days | 28,249 | 26,804 | 23,852 | 25,427 | 19,226* | 22,974 | 21,702* |
| 10 Days | 44,420 | 43,354 | 45,473 | 39,476* | 42,461 | 41,690 | 41,203 |
| Tumour + *C. parvum* Controls (From Day 5 Group) | | 42,511 | 42,896 | 42,613 | 41,667 | 42,121 | 40,104 |

Notes:
(1) Data represent GM of counts/minute of 6 replicates. Limits of 1 standard error have been omitted.
(2) Student's 't' test: *C. parvum*-treated macrophages compared with untreated macrophages.
* $P \ll 0.05$
(3) *C. parvum* itself had no effect on the growth of tumour cells. Data from the Day 5 group only have been presented (see line 6 of table).

*in vitro*. It has been shown that a subcutaneous (Wolmark, Levine and Fisher, 1974) or an intraperitoneal (Baum and Breese, 1976) inoculation of *C. parvum* results in a dramatic yet transient rise in the number of macrophage colony-forming cells from bone marrow. The number of colonies reached a maximum at 2 days suggesting that macrophage precursors may be the initial target in *C. parvum* therapy. Macrophages derived from the bone marrow of tumour-bearing and *C. parvum*/cyclophosphamide-treated tumour-bearing mice exhibited cytotoxicity to tumour cells *in vitro* (Fisher and Wolmark, 1976; Fisher *et al*, 1976b, c).

## 2. ANTI-TUMOUR CYTOTIXICITY OF PERITONEAL MACROPHAGES FOLLOWING SIMULTANEOUS INCUBATION WITH C. PARVUM AND LYMPHOID CELLS FROM C. PARVUM-TREATED MICE

*C. parvum* had failed to activate macrophages on its own and thus a more complex model was envisaged in which macrophages might require the co-operation of T cells or B cells, or both. Evans and Alexander (1972a, b) describe such a mechanism in which lymphocytes from mice sensitised to BCG and other antigens render macrophages from normal mice cytotoxic in the presence of the specific antigen.

It was likely that a similar mechanism operated with *C. parvum* and this possibility was investigated by incubating macrophage monolayers simultaneously with *C. parvum* and glass-incubated spleen cells from mice previously injected intraperitoneally with *C. parvum*. Spleen cells and *C. parvum* were then removed from the monolayers by vigorous washing 24 hours later and replaced with tumour cells to test for cytotoxic activation.

Results from a preliminary experiment were encouraging and further experiments were pursued to ascertain the best protocol for activation. This was achieved in part by empiric means, determining the ideal time course, concentrations of spleen cells and C. parvum to be used per well, and in part by arbitrary selection, for example, of numbers of PE and tumour cells. The formula arrived at for activation (described more fully in Materials and Methods) was (per well): $8 \times 10^4$ PE cells, $4 \times 10^5$ non-adherent spleen cells, and 0.7µg washed C. parvum. The extent of activation was assessed using $4 \times 10^3$ tumour cells per well. This concentration of PEC was selected because it was known from previous work using PEC from C. parvum-treated mice, to give a strong in vitro cytotoxic effect (Ghaffar et al., 1974). The actual adherent effector to tumour target ratio was about 6:1 following the loss of non-adherent PE cells during the washing process.

Table 2 gives the results of a typical experiment using spleen cells from mice injected i.p. 10 days before with 1.4mg C. parvum. It can be seen (line 1, table 2) that only one macrophage treatment resulted in an anti-tumour reaction, namely C. parvum-sensitised spleen cells plus C. parvum. The presence of C. parvum or spleen cells alone was not sufficient to induce activation. The cytotoxic index (CI), compares the effects of normal spleen cells with C. parvum-sensitised spleen cells in the anti-tumour assay: the higher the CI the greater the cytotoxic effect. Where a particular treatment resulted in an increased uptake of $^{125}$IUDR by tumour cells, when compared with the normal controls, this has been represented in tables as a negative cytotoxic index (neg).

TABLE 2 IN VITRO ANTI-TUMOUR CYTOTOXICITY OF NORMAL MACROPHAGES FOLLOWING SIMULTANEOUS INCUBATION WITH C. PARVUM AND SPLEEN CELLS FROM C. PARVUM-TREATED MICE

| *In vitro* Test Group | C. parvum Treatment of Spleen Cell Donors | | C.I. | P(a) |
|---|---|---|---|---|
| | None | *C. parvum* Day -10 | | |
| Spleen + macrophage + *C. parvum* | 17,553(16,475-18,701) | 2,214 (1,770-2,768) | 87 | < 0.001 |
| Spleen + macrophage | 18,862(16,584-21,452) | 30,071 (26,811-33,727) | Neg. | |
| Spleen + *C. parvum* | 18,634(16,521-21,018) | 12,755 (11,133-14,612) | 32 | N.S. |
| Spleen | 16,593(14,420-19,094) | 20,213 (17,410-23,466) | Neg. | |
| Additional Controls | | | | |
| Macrophage + *C. parvum* : | 16,695 (14,771-18,868) | | | |
| Macrophage : | 12,674 (10,649-15,083) | | | |
| Tumour alone : | 16,184 (13,457-19,464) | | | |

(a) Comparison using Student's 't' test of the effects of spleen cells from untreated mice and *C. parvum*-treated mice. P values greater than 0.05 were considered not significant (N.S.)

Several other points should be noted when studying this and subsequent tables:

(1) All presented data, unless otherwise indicated, signify the counts per minute obtained with tumour cells present.

(2) The washing procedure fails to remove all lymphoid cells and thus the possible cytotoxic effects of this residual population have been controlled (eg. lines 2 and 4 in table 2).

(3) All experiments included controls to assess the effects of macrophages with or without *C. parvum*. This is an essential control since there was an everpresent possibility of *in vivo* activation of macrophages as a result of infection in the mouse colony. It was frequently observed that normal unstimulated macrophages actually enhanced the incorporation of DNA into tumour cells.

(4) Macrophages or persistently adherent lymphoid cells do not themselves take up $^{125}$IUDR to any great extent, the counts per minute per well rarely being more than 200 - 300 counts. Such controls have been omitted from the tables.

(5) *C. parvum* itself does not affect the proliferation of tumour cells nor of their uptake of $^{125}$IUDR.

## 3.1 EFFECT OF ROUTE OF ADMINISTRATION AND TIME OF INJECTION OF C. PARVUM ON THE ABILITY OF SPLEEN CELLS TO ACTIVATE MACROPHAGES IN VITRO

In initial experiments *C. parvum* was administered 10 days before harvesting spleen cells. This timing had been selected since it was

known that the in vitro expression of anti-tumour cytotoxicity of PE and other lymphoid cells was at a maximum 4 to 14 days following i.p. injection (Ghaffar and Cullen, 1977). However, it was of interest to know in what way the in vitro activation of macrophages would be affected by different timings and in particular by other routes of administration as it was known that the in vivo anti-tumour effect of C. parvum was dependent on whether it was given subcutaneously, intravenously or intraperitoneally.

Accordingly, groups of mice were injected with C. parvum, either subcutaneously, intraperitoneally or intravenously, at various times (42, 28, 21, 14 and 7 days) before harvesting their spleens for use in the activation assay. The extent of macrophage cytotoxic activation induced by the various spleen samples has been summarised in the form of cytotoxic indices in figure 5. Spleens from the i.p. groups of mice gave good activation up to 21 days following injection. This capacity of i.p. sensitised spleen cells to activate macrophages is not present on day 4 following injection (data not shown) and thus it reaches a maximum very quickly indeed between day 5 and day 7.

The intravenous route led to activation during the first two weeks following injections but by the third week the effect had rapidly disappeared. The subcutaneous route was essentially ineffective as a route for sensitising spleen cells which could activate macrophages.

These results correlate closely with in vivo studies which show that C. parvum causes tumour inhibition when given by the i.p. or i.v. routes but not by the s.c. route (Woodruff and Inchley, 1971;

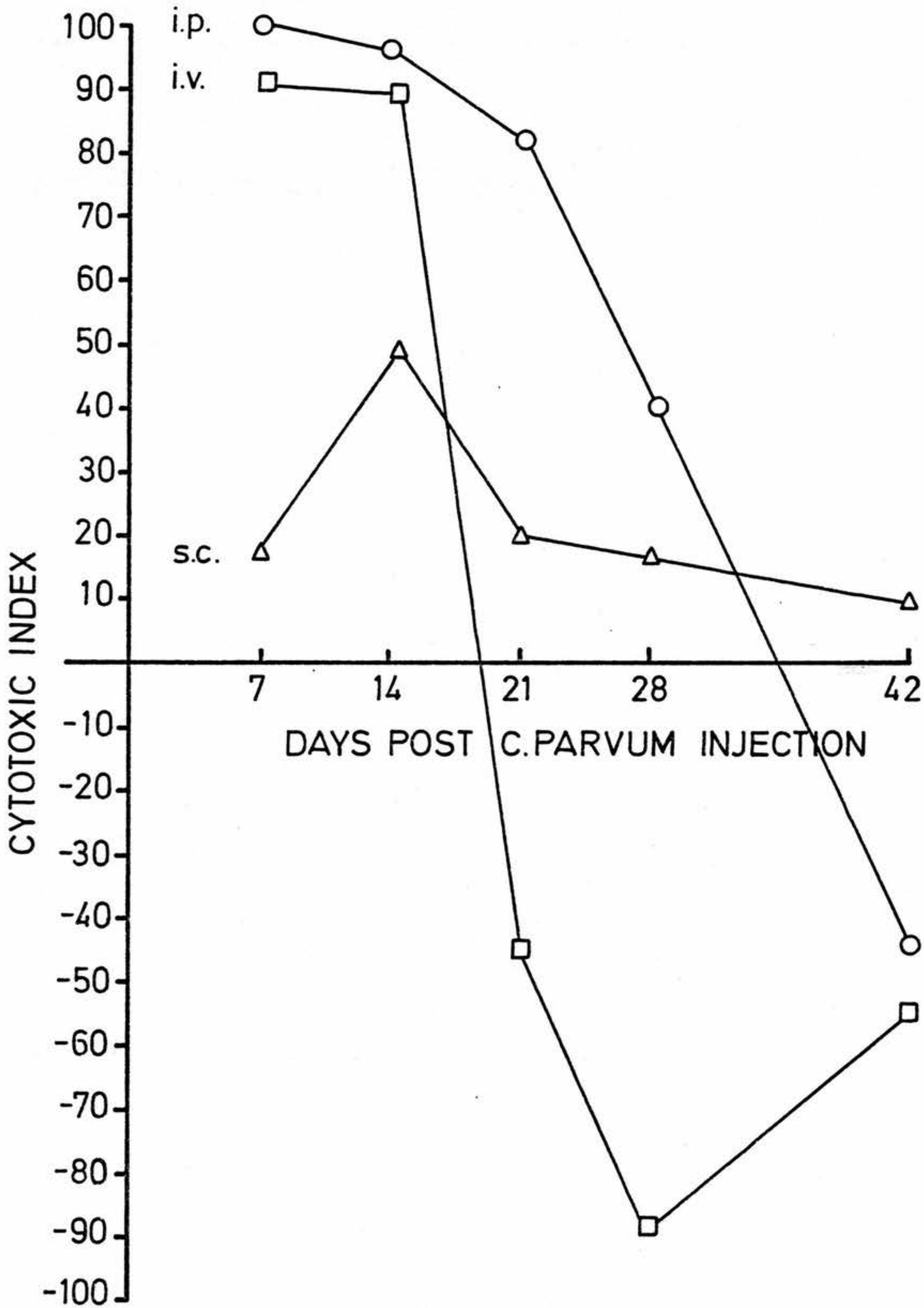



FIGURE 5 EFFECT OF ROUTE OF ADMINISTRATION (ip ○ , iv □ , sc △ ) OF C.PARVUM ON THE CAPACITY OF SPLEEN CELLS TO ACTIVATE MACROPHAGES IN VITRO AND THE VARIATION OF THIS CAPACITY WITH TIME.

Woodruff et al., 1972; Scott, 1974c). It should be noted that antibody titres to C. parvum following i.p. administration are considerably greater than those following s.c. administration (James et al., 1976). In addition, the gradual loss of the capacity of C. parvum-sensitised spleen cells to activate macrophages parallels recent work on the effects of route and time of C. parvum administration on the in vitro anti-tumour effects of lymphoid cells (Krahenbuhl, Lambert and Remington, 1976; Ghaffar and Cullen, 1977). Such correlations might imply that the mechanism of in vitro activation of macrophages being studied could be operating in vivo.

## 3.2 In vitro activation of macrophages by C. parvum and lymph node cells from C. parvum-treated mice

This experiment set out to demonstrate that cells from the lymph nodes of C. parvum-sensitised mice could also activate macrophages in vitro. Mice were injected i.p. or s.c. with 1.4mg C. parvum on day -11. The s.c. inoculation was divided equally between two sites, right foreleg and right hindleg, and only the draining nodes (inguinal, brachial and axillary) from these sites were excised. All inguinal, brachial and axillary nodes were excised in the i.p. and untreated control groups. As with spleen cells, lymph node cells (LNC) were glass-incubated for a total of 3 hours to remove adherent cells before being used in the activation assay. Three concentrations of LNC were used: 16, 8 and 4 x $10^5$ per well (cf. spleen cell concentrations used above of 4 x $10^5$ per well).

The results obtained with 16 x $10^5$ LN cells per well, and shown in table 3(a), indicated that in vitro activation could be achieved

using such cells but that LNC from i.p.-treated animals were not as effective as those from s.c.-treated animals. The comparative ineffectiveness of LNC from the i.p.-treated group may have been because peripheral nodes only were taken; mesenteric node lymphocytes may have given better results. Table 3(b) summarises the results obtained using the lower LNC to macrophage ratios. It can be seen that significant anti-tumour activity was only obtained using the high (16 x $10^5$ LNC per well) ratio, four times as many cells as would be required for a similar effect using spleen cells from i.p.-treated mice.

These results together with those on the effect of route and time of C. parvum administration (figure 5) on in vitro macrophage activation account for the failure of a previous experiment reported by our group (Ghaffar et al., 1975) in which the ratio of LNC to PEC was only 10:1 and not 20:1, and the mice had been injected, not only by the less effective i.p. route, but on day -28, by which time the capacity of the lymphocytes to activate would be greatly diminished.

## 4. CONFIRMATION THAT IN VITRO MACROPHAGE ACTIVATION IS EFFECTED BY THE LYMPHOCYTE POPULATION OF SPLEENS FROM C. PARVUM-TREATED MICE

Experiments so far had shown that spleen cells depleted of glass-adherent cells could activate anti-tumour cytotoxicity in normal macrophages in the presence of C. parvum. It had been assumed at this stage that, (a) the spleen cells involved were lymphocytes and, (b) that they were not directly responsible for the observed cytotoxic effects. However, the possibility could not be excluded that some macrophages or macrophage precursors were surviving the

TABLE 3(a) IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES BY C.PARVUM AND LYMPH NODE CELLS (LNC) FROM C.PARVUM-TREATED MICE: SUBCUTANEOUS AND INTRAPERITONEAL ROUTES

| Treatment of LNC Donor | *In vitro* Test Group | Counts/Min. | CI | P[a] |
|---|---|---|---|---|
| None | LNC[b] + Macrophages + *C.parvum* | 13,102 (10,616-16,171) | | |
| | LNC + Macrophages | 12,183 (11,335-13,094) | | |
| | LNC + *C.parvum* | 19,098 (17,736-20,566) | | |
| | LNC | 18,572 (17,325-19,908) | | |
| *C.parvum* sc[c] day -11 | LNC + Macrophages + *C.parvum* | 3,893 (3,306-4,583) | 70 | <0.005 |
| | LNC + Macrophages | 11,657 (11,312-12,012) | 4 | NS |
| | LNC + *C.parvum* | 12,349 (11,772-12,955) | 35 | <0.005 |
| | LNC | 16,849 (15,652-18,139) | 9 | NS |
| *C.parvum* ip[c] day -11 | LNC + Macrophages + *C.parvum* | 6,470 (6,605-6,901) | 51 | <0.02 |
| | LNC + Macrophages | 11,646 (10,493-12,926) | 4 | NS |
| | LNC + *C.parvum* | 12,660 (11,041-14,516) | 34 | <0.005 |
| | LNC | 13,501 (11,900-15,316) | 27 | NS |
| Additional Controls | Macrophage + *C.parvum* | 10,866(10,423-11,328) | | |
| | Macrophages | 11,700(10,801-12,652) | | |

(a) Comparison of LNC from normal mice with LNC from mice treated sc or ip with *C.parvum*.

(b) LNC used at 16 x $10^5$ per well (cf. experiments using spleen cells, 4 x $10^5$ per well.)

(c) See notes on Table 3(b) for details of *C.parvum* administration.

TABLE 3(b) IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES BY C.PARVUM AND LYMPH NODE CELLS (LNC) FROM C.PARVUM-TREATED MICE:[a] SUMMARY OF DATA USING SUBCUTANEOUS[b] AND INTRAPERITONEAL[c] ROUTES

| Treatment of Macrophage Monolayers | Treatment of Lymph Node Donor | | C.I. | Treatment of Lymph Node Donor | C.I. |
|---|---|---|---|---|---|
| | None | C.parvum sc Day -11 | | C.parvum ip Day -11 | |
| 16 x $10^5$ LNC/well: + C.parvum | 13,102 (10,616-16,171) | 3,893 (3,306-4,583) | 70 | 6,470 (6,065-6,901) | 51 |
| 8 x $10^5$ LNC/well: + C.parvum | 9,083 (8,617-9,574) | 5,695 (4,836-6,706) | 37 | 7,907 (7,491-8,346) | 13 |
| 4 x $10^5$ LNC/well: + C.parvum | 9,594 (9,106-10,108) | 7,543 (6,955-8,180) | 21 | Not done | |
| Other Controls: Macrophages + C.parvum | 10,866 (10,423-11,328) | | | | |
| Other Controls: Macrophages | 11,700 (10,801-12,652) | | | | |

(a) Four mice per group used in this experiment.

(b) Subcutaneous route: 0.7 mg C.parvum (0.1 ml) in each of two sites, right foreleg and right hindleg. Only the draining nodes (inguinal, brachial and axillary) from the two injection sites were excised.

(c) Intraperitoneal route: 1.4 mg C.parvum (0.2 ml) per mouse. All inguinal, brachial and axillary nodes were excised.

glass incubation and that they were either contributing to the cytotoxicity or were the actual activating cells. Several methods involving x-irradiation, gold salt and anti-macrophage serum were employed to investigate these possibilities.

## 4.1 In vitro activation of macrophages: effect of in vitro x-irradiation of spleen cells

The extreme radiosensitivity of lymphocytes and the lymphopoietic system is well recognised (Trowel, 1952; Berdjis, 1971; Anderson and Warner, 1976) and consequently x- or gamma-irradiation has often been employed to kill or inactivate lymphocytes in experimental systems (Ritter *et al*., 1973; Denham *et al*., 1970). Many macrophage functions, on the other hand, are regarded as being radioresistant (Muramatsu, Morita and Sohmura, 1966; Geiger and Gallily, 1974). However, these are generalisations and in reality the cells of the immune system, both lymphocytes and monocyte-macrophages, do contain sub-populations of radiosensitive and radioresistant cells. Furthermore, the sensitivity of a particular immune response depends very much on the circumstances and on the nature of the antigens involved (for review see Taliafero, Taliafero and Jaroslow, 1964; Berdjis, 1971; Anderson and Warner, 1976).

Spleen cells from untreated or *C. parvum*-treated mice were prepared in the usual way but, before incubation on glass, cells were first exposed to x-irradiation doses of 200 or 400 rad.

Data in table 4 reveal a marked reduction in anti-tumour cytotoxicity following exposure of spleen cells to 200r and complete abrogation of cytotoxicity after 400r, indicating that the macrophage

TABLE 4 EFFECT OF THE IN VITRO X-IRRADIATION OF SPLEEN CELLS ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| In Vitro Test Group | In Vitro X-irradiation of Spleen Cells [a] | *C. parvum* treatment of Spleen Cell Donors: None | *C. parvum* treatment of Spleen Cell Donors: *C. parvum* ip Day -8 | C.I. | P [b] |
|---|---|---|---|---|---|
| Spleen Cells + Macrophages + *C. parvum* | None | 13,676(13,091-14,226) | 4,745(3,922-5,742) | 65 | <0.005 |
| | 200 Rad | 9,277(6,655-12,932) | 4,752(4,504-5,013) | 49 | N.S. |
| | 400 Rad | 8,089(7,638-8,567) | 6,531(5,460-7,814) | 19 | N.S. |
| Spleen Cells + Macrophages | None | 15,515(13,467-17,874) | 14,974(14,120-15,880) | 3 | N.S. |
| | 200 Rad | 15,139(12,515-18,314) | 16,658(15,925-17,425) | Neg | |
| | 400 Rad | 11,525(9,617-13,812) | 21,819(21,191-22,465) | Neg | |
| Spleen Cells + *C. parvum* | None | 7,414(6,807-8,076) | 8,308(7,420-9,301) | Neg | |
| | 200 Rad | 5,403(4,844-6,027) | 8,597(7,691-9,610) | Neg | |
| | 400 Rad | 6,081(4,543-8,140) | 6,935(5,812-8,274) | Neg | |
| Spleen Cells | None | 5,753(4,362-7,589) | 9,558(7,322-12,476) | Neg | |
| | 200 Rad | 6,059(4,805-7,642) | 15,497(13,817-17,382) | Neg | |
| | 400 Rad | 5,105(4,026-6,472) | 13,035(12,002-14,157) | Neg | |
| Additional Controls | Macrophage + *C. parvum* | 18,984(18,156-19,850) | | | |
| | Macrophage | 20,746(20,073-21,443) | | | |
| | Tumour Alone | 11,029(10,530-11,552) | | | |

(a) Spleen cells were irradiated prior to incubation on glass

(b) Comparison of spleen cells from normal and *C. parvum*-treated mice; P values greater than 0.05 were considered not significant (N.S.)

activating cell was radiosensitive and thus possibly a lymphocyte or macrophage precursor.

4.2 In vitro x-irradiation of PE cells (Table 5)

In order to eliminate the possibility that peritoneal lymphocytes persisting in wells alongside macrophages were contributing to the activation process, or to the cytotoxic effects directly, PEC were irradiated prior to seeding in microculture plates. It was found that this treatment caused a slight drop in the level of cytotoxicity, and although this drop was significant statistically, it was apparent that the capacity of macrophages to be activated was relatively radioresistant, at least to a dose of 800r. The possibility that radioresistant T helper cells or cytotoxic T cells were still playing a significant part could not be formally excluded but was considered unlikely (see Discussion - effects of x-irradiation).

4.3 Whole-body x-irradiation of spleen cell donor mice

Consistent with the above observation, and further supporting the role of the macrophage as cytotoxic effector cell, are the results reported by Woodruff, Ghaffar and Whitehead (1976b) showing the effects of whole-body and *in vitro* x-irradiation on the *in vitro* anti-tumour activity of PE and spleen cells from *C. parvum*-treated mice. They found that irradiation (whole-body or *in vitro*) failed to abrogate cytotoxicity if activation of effector cells had already occurred. However, whole-body irradiation of 400 rad in the case of PEC and 800 rad in the case of spleen cells, given either 4 days before or immediately before administration of *C. parvum*, completely

TABLE 5 IN VITRO X-IRRADIATION OF PERITONEAL EXUDATE (P.E.) CELLS: EFFECT ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| In Vitro Test Group | In Vitro X-irradiation of Normal P.E. Cells | C. parvum Treatment of Spleen Cell Donors | | C.I. | P[a] | P[b] |
|---|---|---|---|---|---|---|
| | | None | C. parvum ip Day -8 | | | |
| Spleen Cells + Macrophages + C. parvum | None | 16,131(14,277-18,225) | 601(484-746) | 96 | $\ll 0.001$ | |
| | 200 Rad | 17,568(16,671-18,513) | 1,535(1,120-2,106) | 91 | $<0.001$ | $<0.05$ |
| | 800 Rad | 16,609(12,732-21,668) | 2,897(1,945-4,313) | 83 | $<0.02$ | $<0.01$ |
| Spleen Cells + Macrophages | None | 18,944(17,626-20,360) | 20,733(20,142-21,340) | Neg | | |
| | 200 Rad | 12,801(11,282-14,526) | 20,067(17,941-22,444) | Neg | | |
| | 800 Rad | 16,378(15,072-17,797) | 22,293(21,397-23,226) | Neg | | |
| Other Controls | | | | | | |
| Spleen Cells + C. parvum | | 7,535(6,829-8,315) | 4,976(4,390-5,640) | 34 | $<0.05$ | |
| Spleen Cells | | 6,453(5,112-8,147) | 14,746(13,708-15,862) | Neg | | |

(a) Comparison of spleen cells from normal and C. parvum-treated mice.

(b) Effect of irradiation on cytotoxicity: In column 4 comparison of 200 Rad (1,535 counts/minute) and 800 Rad (2,897 counts/minute) with non-irradiation group (601 counts/minute). Irradiation causes a significant drop in cytotoxicity: 200 Rad, $<0.05$ and 800 Rad, $<0.01$.

TABLE 6 EFFECT OF WHOLE-BODY X-IRRADIATION OF SPLEEN CELL DONORS ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| *In vitro* Test Group | X-irradiation of spleen cell donors | *C. parvum* treatment of spleen cell donors: None | *C. parvum* treatment of spleen cell donors: *C.parvum* ip Day -8 | C.I. | P(a) | P(b) |
|---|---|---|---|---|---|---|
| Spleen Cells + Macrophages + *C. parvum* | None<br>200 Rad<br>400 Rad | 21,743(20,270-23,322)<br>21,676(20,368-23,068)<br>18,771(17,473-20,166) | 1,794(1,279-2,515)<br>4,804(4,066-5,675)<br>9,000(7,785-10,406) | 92<br>78<br>52 | ≪0.001<br>≪0.001<br>$<$0.005 | <br>$<$0.05<br>$<$0.005 |
| Spleen Cells + Macrophages | None<br>200 Rad<br>400 Rad | 23,775(23,018-24,557)<br>21,988(19,133-25,270)<br>14,961(12,650-17,695) | 26,430(25,431-27,436)<br>16,265(14,186-18,648)<br>19,559(18,703-20,454) | Neg<br>26<br>Neg | N.S. | |
| Spleen Cells + *C. parvum* | None<br>200 Rad<br>400 Rad | 8,362(6,078-11,505)<br>8,406(7,195-9,821)<br>7,888(6,401-9,720) | 16,293(14,989-17,710)<br>6,816(5,856-7,934)<br>13,990(12,238-15,994) | Neg<br>19<br>Neg | N.S. | |
| Spleen Cells | None<br>200 Rad<br>400 Rad | 10,927(9,313-12,821)<br>9,548(7,987-11,416)<br>7,632(6,748-8,631) | 20,649(17,146-24,867)<br>13,626(11,994-15,481)<br>23,192(22,268-24,153) | Neg<br>Neg<br>Neg | | |
| Additional Controls | | Macrophage + *C. parvum*<br>Macrophage<br>Tumour Alone | 27,962(26,134-29,917)<br>25,136(23,976-26,353)<br>25,347(23,881-26,902) | | | |

(a) Comparison of spleen cells from normal and *C. parvum*-treated mice: P values greater than 0.05 were considered not significant (N.S.)

(b) Effect of irradiation of spleen cells on cytotoxicity: In column 4, comparison of the irradiated groups with the non-irradiated groups shows significant decreases in cytotoxicity

abolished the cytotoxic effects.

The results in table 6 show the effect of in vitro macrophage activation of giving 200 or 400 rad whole-body irradiation to mice one hour before administration of C. parvum. Cytotoxicity was reduced in both irradiated groups but not completely abrogated. This correlates well with the reduction in cytotoxicity of spleen cells in Woodruff et al. (1976b) following whole-body doses of 200 and 400 rad; 800 rad was required to abolish cytotoxicity.

These results using x-irradiation established that the processes in C. parvum-treated mice which led to cytotoxic cells or to spleen cells capable of activating macrophages in vitro, involved a radio-sensitive cell population, but it was not yet clear whether these cells were lymphocytes or macrophage precursors.

## 4.4 Treatment of spleen cells with anti-macrophage serum: no effect on the in vitro activation of macrophages

Although incubation on glass undoubtedly removed the bulk of splenic macrophages, the possibility did exist that a few macrophages, or monocytes, were still present when spleen suspensions were added to peritoneal macrophage monolayers. Such persistent splenic macrophages may have been contributing to the observed anti-tumour effects. To counter this possibility, glass-incubated spleen cells were treated for 1 hour at 37°C with guinea pig serum (as complement source) and either normal rabbit serum, or rabbit anti-macrophage serum (AMS). This thymocyte-absorbed AMS had high specificity for peritoneal macrophages, shown by a 50% cytotoxic titre of 1:512. There was some residual reactivity against thymocytes indicated by a

50% cytotoxic titre of 1:4. AMS was used at a final dilution of 1:64, known to give 100% cytotoxicity against peritonel macrophages but virtually no activity against thymocytes.

The results (table 7(a)) demonstrated that anti-macrophage serum treatment of spleen cells from C. parvum-treated mice did not affect their capacity to activate macrophages *in vitro*. If it is assumed that splenic macrophages and monocytes share an antigen(s) with peritoneal macrophages then this evidence clearly indicates that the observed anti-tumour effects could not be due to macrophages or monocytes persisting in the spleen cell suspensions following glass-adherence. The premise of common antigenicity between macrophages of different sites is supported by several workers. Feldman, Tubergen, Pollock and Unanue (1972) demonstrated in the guinea pig that an anti-peritoneal macrophage serum reacted with up to 50% of splenic macrophages but only 10% peripheral blood monocytes. Montfort and Perez-Tamayo (1971) found a rat peritoneal macrophage antigen(s) common to peripheral monocytes and macrophages in acute inflammatory exudates. An alveolar macrophage serum had specificity for fixed tissue macrophages but not blood monocytes (Martinez and Montfort, 1973).

There is evidence to suggest that anti-macrophage sera are directed at a differentiation antigen(s) associated with cell maturation (Virolainen *et al*., 1971) which would account for the poor specificity of AMS for monocytes reported by Feldman *et al*., (1972) and Martinez and Montfort (1973). The reactivity against monocytes, of the AMS used in the above experiments, is not known, therefore, it is possible that some monocytes could have reached the microculture

TABLE 7(a) IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

FAILURE OF ANTI-MACROPHAGE SERUM TREATMENT OF SPLEEN CELLS TO ABROGATE ACTIVATION

| Serum Treatment of Spleen Cells [a] | *In Vitro* Test Group | Spleen Cell Donor Treatment | | C.I. | P [b] |
|---|---|---|---|---|---|
| | | None | *C.parvum* ip Day -11 | | |
| Normal Rabbit Serum + Guinea Pig Complement | Spleen + Macrophage + *C. parvum* | 52,131(50,182-54,156) | 9,040(8,338-9,802) | 83 | ≪0.001 |
| | Spleen + Macrophage | 51,235(49,242-53,308) | 49,143(46,773-51,632) | 4 | N.S. |
| | Spleen + *C.parvum* | 51,399(48,203-54,806) | 38,115(36,563-39,734) | 26 | <0.01 |
| | Spleen | 38,210(35,371-41,276) | 49,384(47,061-51,823) | Neg | |
| Rabbit Anti-Macrophage Serum + Guinea Pig Complement | Spleen + Macrophage + *C. parvum* | 43,100(40,585-45,770) | 8,330(7,137-9,722) | 81 | <0.001 |
| | Spleen + Macrophage | 43,931(43,058-44,821) | 52,713(51,481-53,975) | Neg | |
| | Spleen + *C.parvum* | 45,726(42,757-48,901) | 42,033(40,765-43,341) | 8 | N.S. |
| | Spleen | 44,794(40,449-49,606) | 50,968(49,569-52,407) | Neg | |
| Guinea Pig Complement | Spleen + Macrophage + *C. parvum* | 57,050(56,658-57,445) | 12,811(11,083-14,809) | 78 | <0.001 |

(a) Following incubation on glass for 3 hours, spleen cells were incubated at 37°C for 1 hour in the presence of guinea pig serum (as complement source) and either normal rabbit serum, or rabbit anti-macrophage serum. Final serum dilutions used were 1:64 for normal rabbit and rabbit anti-macrophage, and 1:16 for guinea pig serum.

(b) Comparison of spleen cells from normal and *C. parvum*-treated mice. P values greater than 0.05 were considered not significant.

TABLE 7(b) EFFECT OF ANTI-MACROPHAGE SERUM AND COMPLEMENT ON THE ANTI-TUMOUR CYTOTOXICITY OF SPLEEN CELLS FROM C.PARVUM-TREATED MICE: SPLEEN CELLS ADDED DIRECTLY TO TUMOUR CELL MONOLAYERS[a]

| Serum Treatment[b] of Spleen Cells | Effector to Target Ratio | Treatment of Spleen Cell Donor | | C.I. |
|---|---|---|---|---|
| | | None | *C.parvum* ip Day -10 | |
| None | 400 : 1 | 27,635(26,011-29,359) | 265 (243-288) | 99 |
| | 200 : 1 | 43,337(42,372-44,324) | 281 (269-294) | 99 |
| | 100 : 1 | 50,865(49,850-51,902) | 704 (681-729) | 99 |
| Normal rabbit serum + Guinea pig complement | 400 : 1 | 53,638(52,587-54,710) | 3,247(3,209-3,286) | 94 |
| | 200 : 1 | 57,193(55,767-58,655) | 25,283(24,054-26,574) | 56 |
| | 100 : 1 | 59,944(58,922-60,983) | 44,292(43,162-45,452) | 26 |
| Anti-macrophage serum + Guinea pig complement | 400 : 1 | 55,103(54,261-55,958) | 18,199(17,084-19,387) | 67 |
| | 200 : 1 | 56,527(54,639-58,480) | 40,889(39,589-42,233) | 28 |
| | 100 : 1 | 63,987(62,776-65,221) | 51,289(49,395-53,255) | 20 |
| Tumour Cell Control | | 64,196 (63,557-64,842) | | |

(a) Treated and untreated spleen cells added to tumour cell monolayers ($4 \times 10^3$ cells/well) and cytotoxicity assessed 48 hours later.

(b) Spleen cells treated with normal rabbit or anti-macrophage serum at a final dilution of 1:64 together with guinea pig serum at a final dilution of 1:16 for 1 hour at $37^{\circ}C$.

wells.

The effect of anti-macrophage serum on the direct cytotoxicity of spleen cells to tumour cells *in vitro* is shown in table 7(b). Treatment with guinea pig complement and either normal rabbit serum, or AMS, markedly reduced the anti-tumour activity of spleen cells from *C. parvum*-treated mice, particularly at the lower effector to target ratios. These results indicate that the macrophage population of spleen cells was being effectively eliminated by the serum pre-treatments and thus confirmed that macrophages were not the activating cell type.

## 4.5 In vitro activation of macrophages: effect of gold salt treatment of spleen cell donors

Gold salts, recognised anti-inflammatory compounds (Walz *et al*., 1974), are known to localise in the reticuloendothelial system and at sites of inflammation (Swartz *et al*., 1960; Persellin and Ziff, 1966). Lysosomes are actively involved in the destruction of articular cartilage in rheumatoid arthritis (Dingle, 1969; Chayen and Bitensky, 1971) and it has been postulated that the therapeutic effects of gold salts may be due to the inactivation of macrophage lysosomal enzymes. Ennis *et al*., (1968) demonstrated that gold thiomalate and gold thioglucose could inhibit the lysosome enzymes acid phosphatase, β-glucuronidase and cathepsin, by reversibly binding to enzyme sulphydryl groups, but did not affect actual release of the enzymes from lysosomes. It has also been shown that sodium aurothiomalate suppresses phagocytosis and migration of macrophages, (Jessop *et al*., 1973; Vernon-Roberts *et al*., 1973).

These effects of gold compounds on macrophages have been used to advantage in studies aimed at elucidating the role of macrophages in the anti-tumour effect of C. parvum (James et al., 1976; McBride et al., 1975b; Ghaffar et al., 1976b). It was, therefore, natural to extend this work to investigate the effects of gold salt on the in vitro activation of macrophages.

Normal mice and C. parvum-treated mice were given multiple 1 mg doses of sodium aurothiomalate (as described in Materials and Methods) and their spleens were excised and processed in the usual way, for testing in the activation assay. Table 8 shows that in vivo gold treatment completely abrogates the stimulation by C. parvum of spleens capable of activating macrophages in vitro. This result is in complete agreement with previous work showing the antagonism of gold salt treatment for C. parvum's anti-tumour effects, whether measured in vivo (McBride et al., 1975b) or in vitro (Ghaffar et al., 1976b).

## 4.6 In vitro activation of macrophages: effect of the presence of gold salt in vitro

The influence of in vitro sodium aurothiomalate on the activation of normal macrophages by C. parvum and C. parvum-sensitised spleen cells was investigated. The gold compound was added at one of three stages of the activation assay: during initial macrophage adherence; during activation; and during the incubation of spleen cells to remove the glass-adherent population. The gold was removed by thorough washing before proceeding with the next stage of the assay. This

TABLE 8 EFFECT OF GOLD SALT TREATMENT OF SPLEEN CELL DONORS ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| *In vivo* gold salt treatment | *In vitro* test group | Spleen Cell Donor Treatment | | C.I. | P[b] |
|---|---|---|---|---|---|
| | | None | *C. parvum* ip Day -7 | | |
| None | Spleen + Macrophage + *C. parvum* | 6,626(6,253-7,021) | 178(173-184) | 97 | <<0.001 |
| | Spleen + Macrophage | 5,014(4,751-5,291) | 8,532(8,286-8,784) | Neg | |
| | Spleen + *C. parvum* | 2,313(2,022-2,645) | 2,370(2,062-2,725) | Neg | |
| | Spleen | 2,657(2,470-2,860) | 4,008(3,391-4,738) | Neg | |
| 4[a] x 1mg doses gold salt ip | Spleen + Macrophage + *C. parvum* | 10,261(9,655-10,906) | 8,882(8,088-9,754) | 13 | N.S. |
| | Spleen + Macrophage | 5,961(5,385-6,598) | 3,607(3,348-3,886) | 39 | <0.005 |
| | Spleen + *C. parvum* | 3,288(3,057-3,537) | 9,010(8,266-9,821) | Neg | |
| | Spleen | 3,736(3,258-4,284) | 2,520(2,252-2,820) | 33 | N.S. |
| Additional Controls | Macrophage + *C.parvum* | 10,020(9,485-10,584) | | | |
| | Macrophage | 6,010(5,726-6,309) | | | |
| | Tumour Alone | 5,450(4,906-6,054) | | | |

(a) 1mg sodium aurothiomalate administered ip on days -8, -6, -4 and -1

(b) Comparison of normal and *C. parvum*-treated groups; P values greater than 0.05 were considered not significant

was particularly important in those groups receiving gold salt in the microculture wells since it was known that this compound is very toxic for the fibrosarcoma tumour cells being used.

The results obtained with the various treatments are summarised in table 9 (some of the control data have been omitted for sake of clarity). There was no anti-tumour cytotoxicity if sodium aurothiomalate had been present during macrophage adherence or during the activation process. Prior treatment of spleen cells with gold salt had no effect, lending further support to the anti-macrophage serum evidence that splenic monocytes or macrophages that escape the glass-adherence procedure do not contribute significantly to the cytotoxic effects, either directly or as activators of normal macrophages. These results demonstrated that sodium aurothiomalate exerts its effects at the level of the macrophage, both _in vitro_ and _in vivo._ However, it was not clear whether gold salt interferes with the expression of macrophage cytotoxicity or with the activation process itself (_in vitro_ and _in vivo_). The former seems more likely in view of the observation that the _in vitro_ cytotoxicity of PEC stimulated by _C. parvum_ _in vivo_ could be blocked by gold salt (Ghaffar _et al._, 1976b). The mechanism of this abrogation may depend on the inhibition of lysosomal enzymes (as discussed above) which may mediate the observed effects. It has been shown that the cytotoxic effects of BCG-stimulated macrophages can be inhibited by stabilising lysosome membranes with hydrocortisone or by inactivating the lysosomal enzymes before they are exocytosed into target cells (Hibbs, 1974).

TABLE 9 EFFECT OF GOLD SALT ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| In Vitro Gold Salt Treatment | Macrophages + *C. parvum* incubated with | | C.I. | P [a] |
|---|---|---|---|---|
| | Normal Spleen Cells | *C. parvum* Spleen Cells | | |
| None | 49,442(44,625-54,780) | 26,904(25,658-28,211) | 46 | <0.005 |
| 25µg/well during macrophage adherence | 32,276(27,361-38,074) | 38,745(35,803-41,929) | Neg | |
| 25µg/well during activation | 46,168(41,348-51,550) | 44,460(42,712-46,280) | 4 | N.S. |
| 2500µg/$10^7$ spleen cells in 10ml during glass incubation | 62,808(61,061-64,605) | 34,554(31,312-38,131) | 45 | ≤0.005 |

| Additional Controls | | |
|---|---|---|
| | Macrophage + *C. parvum* + Gold | 52,075(49,735-54,524) |
| | Macrophage + *C. parvum* | 53,893(49,538-58,632) |
| | Tumour Alone | 46,328(40,890-52,489) |

(a) Comparison of normal and *C. parvum*-treated groups: P values greater than 0.05 were considered not significant (N.S.).

## 5. IN VITRO ACTIVATION OF NORMAL MACROPHAGES: FURTHER CHARACTERISATION OF THE MECHANISM

The following experiments were designed to determine (a) the identity of the activating cells, and (b) the manner in which activation was effected, i.e. whether by cell-to-cell contact or by a soluble factor(s) released into the culture medium.

### 5.1 Identification of the activating lymphocytes

The bulk of evidence would favour a role for T cells, both in systems measuring activation by intracellular killing of microbes or, by anti-tumour cytotoxicity. It has been shown that resistance to infection with *Listeria monocytogenes* is effected through a collaboration between antigen-stimulated lymphoid cells and macrophages which could then kill, in a non-specific way, intracellular organisms (Mackaness, 1969; Blanden, Lefford and Mackaness, 1969). The co-operating cell was later identified as a T cell(Lane and Unanue, 1972; Blanden and Langman, 1972; North, 1973).

Similar conclusions were reached by Evans and Alexander (1970, 1972 a, b) using an anti-tumour system. They found that normal macrophages could be activated to specific growth inhibition of target lymphoma cells *in vitro*, either by direct contact of hyperimmune lymphoid cells and macrophages, or by incubating macrophages with a factor from mixed cultures of lymphoma-immune spleen cells and the specific lymphoma cells. This factor was called specific macrophage arming factor (SMAF). Similar results in allogeneic systems have now been described by other groups (Lohmann-Matthes, Ziegler and Fischer, 1973; Zembala, Ptak and Hanczakowska, 1973; Pels and Den

Otter, 1974). Of particular relevance to this thesis is the work demonstrating the *in vitro* stimulation of non-specific cytotoxicity in macrophages by supernatant factors derived from culturing lymphoid cells immune to BCG (Evans, Cox and Alexander, 1973; Piessens, Churchill and David, 1975) or *C. parvum* (Christie and Bomford, 1975) with the specific immunising antigen.

As in the bacterial systems, macrophage activation was found to be a T cell-dependent phenomenon (Evans *et al*., 1972; Grant *et al*., 1973; Lohmann-Matthes *et al*., 1973; Christie and Bomford, 1975). However, there have been reports of macrophage activation systems in which B lymphocytes have played a role (Wilton, Rosenstreich and Oppenheim, 1975; Yamazaki, Shinoda and Mizuno, 1975).

In order to delineate the possible roles of B cells and T cells in the activation of macrophages by *C. parvum*, experiments were set up in which spleen cells from *C. parvum*-treated mice were either treated with anti-Thy-1.2 serum to remove T cell activity or passed through nylon wool columns to remove immunoglobulin-bearing cells (B cells). Experiments using spleen cells from *C. parvum*-treated thymectomised-irradiated-bone marrow reconstituted mice were also set up.

## 5.2 Anti-Thy-1.2 serum treatment of spleen cells

Spleen cells from untreated and *C. parvum*-treated mice were depleted of glass-adherent cells and incubated with anti-Thy-1.2 serum and guinea pig serum as complement source. The effect of this treatment on the capacity of *C. parvum*-immune spleen cells to activate normal macrophages is given in detail in table 10 from one such

TABLE 10 IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES BY C.PARVUM AND SPLEEN CELLS FROM C.PARVUM-TREATED MICE: EFFECT OF TREATING SPLEEN CELLS WITH ANTI-THY 1.2 SERUM AND GUINEA PIG COMPLEMENT(C')

| In vitro Test Group | Serum Treatment of Spleen Cells [a] | C.parvum Treatment of Spleen Cell Donors: None | C.parvum Treatment of Spleen Cell Donors: C.parvum ip Day -11 | C.I. | P [b] |
|---|---|---|---|---|---|
| Spleen cells + Macrophages + C.parvum | None | 54,290(53,408-55,186) | 19,245(17,693-20,932) | 65 | <0.001 |
| | Normal AKR + C' | 52,393(51,931-52,860) | 29,053(26,939-31,334) | 45 | <0.001 |
| | Anti-Thy 1.2 + C' | 60,148(59,347-60,959) | 31,927(30,191-33,762) | 47 | <0.001 |
| | Complement (C') | 57,050(56,658-57,445) | 12,811(11,083-14,809) | 78 | <0.001 |
| Spleen cells + Macrophages | None | 59,306(56,550-62,196) | 61,510(58,890-64,246) | Neg | |
| | Normal AKR + C' | 57,292(56,140-58,467) | 52,327(49,931-54,838) | 8·7 | N.S. |
| | Anti-Thy 1.2 + C' | 55,830(54,488-57,205) | 54,945(53,095-56,859) | 1·6 | N.S. |
| Spleen cells + C.parvum | None | 41,284(36,593-46,577) | 44,913(43,815-46,038) | Neg | |
| | Normal AKR + C' | 35,820(31,735-40,430) | 47,540(46,324-48,788) | Neg | |
| | Anti-Thy 1·2 + C' | 50,274(47,632-53,062) | 55,146(54,358-55,945) | Neg | |
| Spleen cells | None | 44,686(41,447-48,178) | 45,562(41,594-49,908) | Neg | |
| | Normal AKR + C' | 46,126(43,127-49,332) | 47,586(43,048-52,602) | Neg | |
| | Anti-Thy 1·2 + C' | 53,198(49,260-57,451) | 53,516(51,653-55,446) | Neg | |
| Additional Controls | Macrophage + C.parvum | 62,747(62,478-63,016) | | | |
| | C.parvum | 60,816(59,628-62,026) | | | |

(a) See Materials and Methods section for details of serum treatment.

(b) Comparison of spleen cells from normal and C.parvum-treated mice: P values greater than 0.05 were considered not significant (N.S.).

TABLE 11 EFFECT OF TREATING SPLEEN CELLS WITH ANTI-THY-1.2 SERUM ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES: SUMMARY OF RESULTS FROM 4 EXPERIMENTS

| Serum Treatment of Spleen Cells | Cytotoxic Indices from Spleen-Macrophage-*C.parvum* Group | | | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2[a] | Expt. 3 | Expt. 4 |
| None | 90 | 68 | 65 | 70 |
| Normal AKR Serum | 35 | 36 | 45 | 45 |
| Anti-Thy-1.2 Serum | 45 | 55 | 47 | 26 |
| G. Pig Complement | 77 | 57 | 78 | 61 |

[a] In this experiment a commercial preparation of anti-Thy-1.2 serum was used at a final dilution of 1 : 64 (G.D. Searle and Co. Ltd., High Wycombe, England).

experiment, and in summary from other experiments in table 11. Activation was reduced in all experiments by anti-Thy-1.2 serum treatment but, unfortunately, the results were difficult to analyse since normal serum treatment was generally as effective, and in some experiments more so, in ablating activation. This effect of normal serum was surprising since, at the dilution used, this serum was known to exhibit virtually no toxicity to normal CBA spleen cells and thymocytes. These results might be explained by postulating that a non-T cell population was also involved in activation and that serum treatment, if not actually killing these cells, was interfering with their function, possibly by blocking receptors.

## 5.2 Effect of T cell deprivation of spleen cell donor mice

Spleen cells from immunologically intact and T cell-deprived mice were tested in the activation assay at various times (3, 6, 8 and 12 days) following i.p. C. parvum injection. Results using spleens taken 8 days after C. parvum (table 12) show that lymphocytes in T cell-deprived mice do not acquire the capacity to activate macrophages *in vitro*. The time course of activation by spleen cells is summarised in figure 6 as cytotoxic indices. By 12 days post C. parvum injection spleen cells from T cell-deprived mice can cause some activation of cytotoxicity. It is tantalising to speculate that activation might have been greater had spleens been harvested several days later. This slight activation with day 12 spleen cells may reflect the presence in T cell-deprived mice of a residual T cell population which may even be expanded in the response to C. parvum. These results parellel closely those reported by Bomford

TABLE 12 EFFECT OF T CELL DEPRIVATION[a] OF SPLEEN CELL DONORS ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES

| Mice | *In Vitro* Test Group | Spleen Cell Donor Treatment: None | Spleen Cell Donor Treatment: *C. parvum* ip Day -8 | C.I. | P[b] |
|---|---|---|---|---|---|
| Normal | Spleen + Macrophage + *C. parvum* | 14,011(11,588-16,942) | 2,565(2,373-2,773) | 82 | $\ll$0.001 |
| | Spleen + Macrophage | 18,648(17,247-20,162) | 15,425(14,433-16,486) | 17 | N.S. |
| | Spleen + *C. parvum* | 6,920(6,451-7,423) | 5,030(4,025-6,286) | 27 | N.S. |
| | Spleen | 9,944(8,926-11,079) | 10,949(10,305-11,634) | Neg | |
| T Cell Deprived | Spleen + Macrophage + *C. parvum* | 10,924(9,323-12,800) | 8,494(7,257-9,942) | 22 | N.S. |
| | Spleen + Macrophage | 13,078(11,561-14,795) | 12,247(11,193-13,399) | 6 | N.S. |
| | Spleen + *C. parvum* | 5,447(4,604-6443) | 8,861(6,926-11,336) | Neg | |
| | Spleen | 3,963(3,223-4,873) | 6,836(5,652-8,268) | Neg | |
| Other Controls | Macrophage + *C.parvum* | 18,984(18,156-19,850) | | | |
| | Macrophage | 20,746(20,073-21,443) | | | |

(a) See Materials and Methods section for details of this procedure

(b) Comparison of spleen cells from untreated mice and *C. parvum*-treated mice P values greater than 0.05 were considered not significant

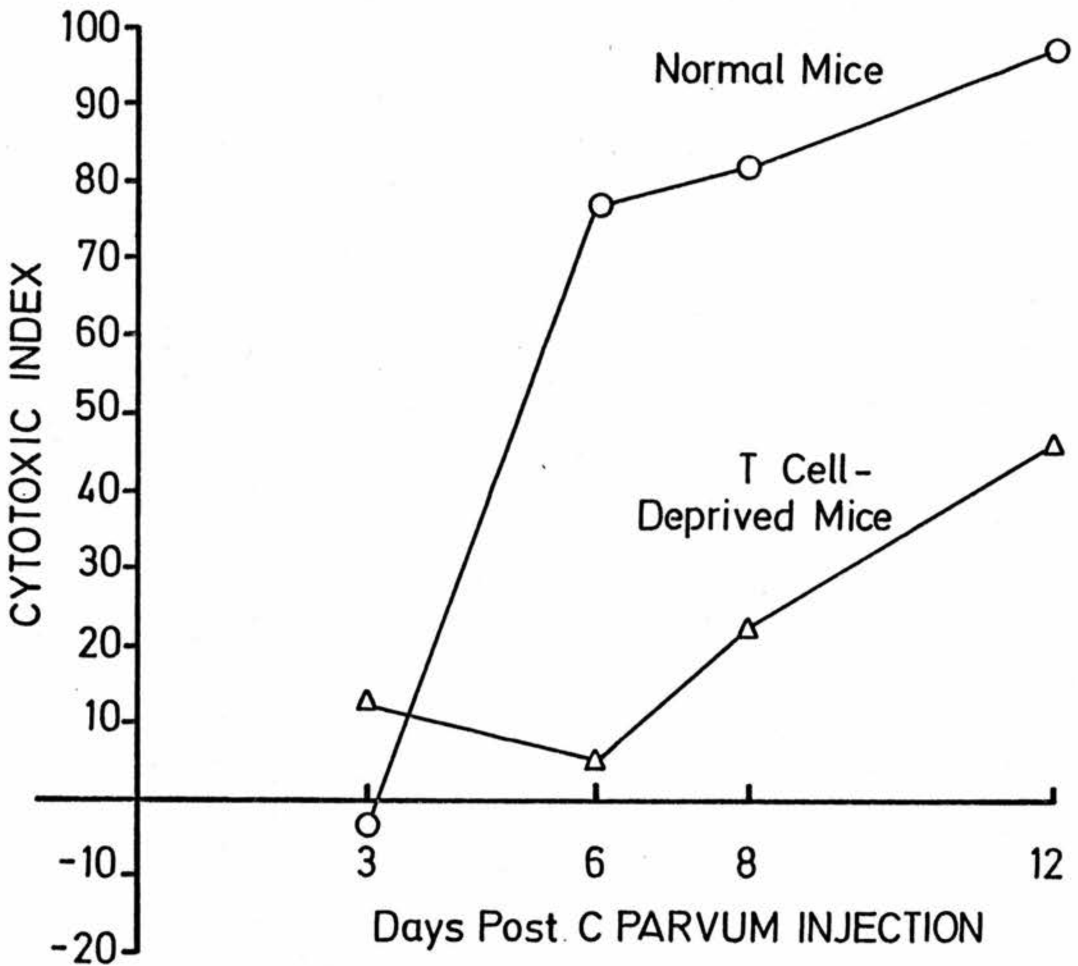



FIGURE 6 IN VITRO ACTIVATION OF MACROPHAGES: EFFECT OF T CELL-DEPRIVATION OF SPLEEN CELL DONORS.

and Christie (1975) using a similar test system.

It is of interest that *in vivo* activation of macrophages by *C. parvum* is not affected by T cell depletion (Ghaffar *et al*., 1975; Bomford and Christie, 1975). This was confirmed by assessing spleen cells, which had not been glass-incubated, for direct cytotoxicity to tumour target cells.

$4 \times 10^3$ tumour cells were seeded in wells of microculture plates and, after 1½ hours incubation at 37°C, spleen cells at various effector to target ratios were added to the wells. 48 hours later plates were shaken to resuspend the bulk of the spleen cells which were then removed. Cytotoxicity was then assessed by incubating the tumour cells with $^{125}$IUDR for 20 hours. Anti-tumour potential appears by day 3 in both normal and T-deprived mouse spleens but reaches a peak first (day 6) inthe latter group (table 13). By day 12 the activity is declining in T cell-deprived mice and at a maximum in the normal group. It was obvious from this experiment and others (Ghaffar *et al*., 1974) that *in vivo* activation by *C. parvum* is an early event differing in its time course to that of the development of lymphocyte potential to activate *in vitro*. A possible explanation of why spleen cells in T cell-deprived mice reached a peak of cytotoxicity earlier than those in T cell-normal mice, could be that the T cell-deprived mice were simply deficient in suppressor T cells.

## 5.4 Nylon wool column depletion of spleen immunoglobulin (Ig)-bearing cells

Separation of non-glass-adherent spleen cells into B cell depleted and B cell enriched fractions was achieved by two consecutive

TABLE 13 ANTI-TUMOUR CYTOTOXICITY OF SPLEEN CELLS FROM C. PARVUM-TREATED T CELL DEPRIVED MICE AND C. PARVUM-TREATED NORMAL MICE. SPLEEN CELLS ADDED DIRECTLY TO TUMOUR CELL MONOLAYERS[(a)]

| Mice | Effector:Target Cell Ratio | ip *C. parvum* Treatment of Spleen Cell Donors | | | | Cytotoxic Index | | |
|---|---|---|---|---|---|---|---|---|
| | | None | C.parvum Day -3 | C. parvum Day -6 | C.parvum Day -12 | Day -3 | Day -6 | Day -12 |
| Normal | 200:1 | 4,380[(b)] | 2,371 | 317 | 142 | 46 | 93 | 97 |
| | 100:1 | 11,054 | 9,492 | 1,430 | 157 | 14 | 87 | 99 |
| | 50:1 | 24,837 | 21,708 | 4,795 | 535 | 13 | 81 | 99 |
| T Cell Deprived | 200:1 | 7,179 | 771 | 499 | 1,110 | 89 | 93 | 85 |
| | 100:1 | 14,870 | 5,499 | 2,468 | 3,619 | 63 | 83 | 76 |
| | 50:1 | 23,515 | 14,713 | 8,457 | 10,175 | 37 | 64 | 57 |

(a) Tumour cells seeded in wells ($4 \times 10^3$ cells/well) 1.5 hours before the addition of spleen cells. After 48 hours the plate was shaken and the wells washed once before the addition of $^{125}$IUDR. Isotope incorporation was counted 18 hours later.

(b) In the interests of clarity, geometric means of 4 counts are presented without their standard errors.

incubations in nylon wool columns. The effluent cell population from such columns has been shown to be greatly depleted in B precursor and memory cell activity, but to be enriched with T cells having helper and cytotoxic effector cell functions. (Julius et al., 1973; Handwerger and Schwartz, 1974; Trizio and Cudkowicz, 1974). Nylon-adherent cells provide a B cell enriched fraction that can be recovered by agitation and thorough washing of the wool (Handwerger and Schwartz, 1974). The relative numbers of Ig-bearing cells in the two separated populations and in the original untreated spleen populations were assessed using an indirect immunofluorescence staining method for cells carrying surface immunoglobulin light chains (table 14).

Table 14

| Spleen cell fraction | % cells bearing Ig light chains | |
|---|---|---|
| | Normal spleen cells | C. parvum spleen cells |
| Untreated | 35 | 39 |
| B cell depleted | 8 | 7 |
| B cell enriched | 60 | 60 |

B cell depleted and B cell enriched fractions were compared with unseparated spleen cells in the activation assay. It was found that depletion of the spleen B cell population greatly reduced macrophage-mediated cytotoxicity (table 15). It was also found that the nylon-adherent (B cell enriched) cells had retained the capacity to induce macrophage-mediated cytotoxicity. These results thus strongly implicated a role for B cells in the activation process.

TABLE 15 EFFECT OF PASSING SPLEEN CELLS THROUGH NYLON WOOL COLUMNS ON THE IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES BY C.PARVUM AND SPLEEN CELLS FROM C.PARVUM-TREATED MICE

| In Vitro Test Group | Treatment of Spleen Cells | C.parvum treatment of spleen cell donors | | CI | P(a) |
|---|---|---|---|---|---|
| | | None | C.parvum ip Day -10 | | |
| Spleen cells + Macrophages + C.parvum | None | 28,126(26,889-29,419) | 6,747(6,084-7,483) | 76 | <<0.001 |
| | B cell depletion | 26,748(25,294-28,285) | 17,550(15,900-19,371) | 34 | <0.01 |
| | B cell enrichment | 24,949(24,294-25,623) | 8,118(7,115-9,262) | 67 | <0.001 |
| Spleen cells + Macrophages | None | 29,524(29,196-29,856) | 25,463(24,579-26,379) | 14 | <0.01 |
| | B cell depletion | 26,126(24,405-27,969) | 22,154(20,878-23,508) | 15 | NS |
| | B cell enrichment | 24,246(22,332-26,323) | 24,570(23,834-25,329) | Neg | |
| Spleen cells + C.parvum | None | 25,669(23,993-27,462) | 17,745(17,413-18,084) | 31 | <0.005 |
| | B cell depletion | 22,024(21,320-22,751) | 21,501(21,012-22,001) | 2 | NS |
| | B cell enrichment | 23,502(23,031-23,982) | 18,627(17,602-19,712) | 21 | <0.01 |
| Spleen cells | None | 28,232(25,296-31,508) | 30,055(28,010-32,250) | Neg | |
| | B cell depletion | 23,373(21,847-25,005) | 35,116(32,535-37,902) | Neg | |
| | B cell enrichment | 22,912(22,197-23,651) | 34,982(33,172-36,891) | Neg | |
| Additional Controls | Macrophage + C.parvum | 31,814(29,973-33,768) | | | |
| | C.parvum | 28,074(27,049-29,136) | | | |

(a) Comparison of spleen cells from untreated and C.parvum-treated mice. P values greater than 0.05 were considered not significant.

However, since the separated fractions were not pure populations of T cells and B cells it could not be concluded that B cells were involved to the exclusion of other cell types.

A valid criticism of these results would be that no functional tests were performed for B cells or T cells. It was assumed from the work of others (*vide supra*) using similar methods and materials that nylon wool separation did not affect the immunological competence of lymphocytes. However, as a recent report has shown, some changes do occur, at least in nylon wool treated thymocytes, such as altered surface morphology and depressed cyclic adenosine monophosphate levels (Kwock, Lin and Wallach, 1976).

Possible mechanisms by which B cells could induce macrophage-mediated cytotoxicity might be that B cells release a macrophage activating lymphokine or that they produce antibody in response to *C. parvum* which then either activates macrophages through the formation of antigen/antibody complexes, or binds to tumour cells, thus allowing an antibody-dependent macrophage killing of target cells. James and his colleagues (1976, 1977), have shown that immunoglobulins produced in *C. parvum*-treated mice are capable of binding to MC-induced fibrosarcoma cells *in vitro*. The implications of these B cell results are explored further in the discussion.

## 5.5 Macrophage activation mediated by factors from spleen cells

It was not clear whether the co-operation between *C. parvum*-sensitised lymphocytes and normal macrophages was a process requiring cell-to-cell contact or could be mediated by a soluble factor or factors released into the culture medium. To resolve this, non-

glass-adherent spleen cells from normal and _C. parvum_-treated mice were cultured at 37°C with or without washed _C. parvum_. Cell free supernatants were prepared after 24 hours culture, by passage through 0.22µm Millipore filters. 0.2 ml volumes of these supernatants were added to normal macrophage monolayers. 0.7 µg washed _C. parvum_ was added to some of the microculture wells. 24 hours later macrophage monolayers were washed once and tumour cells added to assess cytotoxicity.

Macrophages treated with supernatants from _C. parvum_-immune spleen cells alone showed slightly increased cytotoxicity compared to those treated with normal spleen cell supernatant, but this was not significant in a Student's 't'-test (Table 16). Thus, only the supernatant derived from _C. parvum_-immune spleen cells cultured with _C. parvum_ was able to activate normal macrophages. The presence of _C. parvum_ was not required in the microculture wells for activation to occur. This apparent non-requirement for the addition of the specific antigen during activation has also been reported by Evans _et al_., (1972) using BCG stimulated lymphoid cells. This is in contrast to the lymphoma system described by Evans and Alexander in which macrophages, exposed to supernatant factors derived from the _in vitro_ interaction of lymphoma-immune lymphocytes and the specific lymphoma cells used for immunisation, were subsequently cytotoxic to that particular lymphoma only. But if macrophages were exposed to both the supernatant factors and the specific lymphoma cells, then a state of non-specific cytotoxicity was induced in which any target cell could be affected. (Evans and Alexander, 1970; 1972a, b). A likely explanation for this apparent discrepancy

TABLE 16 STIMULATION OF ANTI-TUMOUR CYTOTOXICITY IN NORMAL MACROPHAGES BY SUPERNATANTS FROM CULTURES OF SPLEEN CELLS FROM C. PARVUM-TREATED MICE

| *C. parvum* During Macrophage Activation | Preparation of Cell-Free Supernatants | | | C.I. | P[a] |
|---|---|---|---|---|---|
| | Medium | Normal Spleen Cells | *C.parvum* Spleen Cells | | |
| - | 7,456(6,552-8,486) | 8,445(8,269-8,625) | 5,637(4,229-7,515) | 33 | N.S. |
| + | 7,438(5,995-9,228) | 6,271(5,965-6,593) | 3,954(3,081-5,074) | 37 | N.S. |
| *C. parvum* During Macrophage Activation | Medium + *C.parvum* | Normal Spleen Cells + *C.parvum* | *C.parvum* Spleen Cells + *C.parvum* | C.I. | P[a] |
| - | 7,802(6,831-8,912) | 7,431(7,111-7,766) | 1,407(1,313-1,507) | 81 | $\ll 0.001$ |
| + | 6,675(5,775-7,714) | 7,323(6,295-8,519) | 1,188(1,021-1,382) | 84 | $<0.001$ |

(a) Comparison of the supernatant from cultures of normal spleen cells with the supernatant from cultures of spleen cells from *C. parvum*-treated mice.

between the lymphoma system and the BCG and _C. parvum_ systems would be that, in the latter systems, culture supernatants also contain soluble BCG (or _C. parvum_) antigens which then serve in the activation process.

However, in similar experiments Christie and Bomford (1975) found that _C. parvum_-immune spleen cells when cultured produce a factor which would activate macrophages, but that the greatest cytotoxic effects were achieved when _C. parvum_ had been present, both in the spleen cell cultures, and during activation. These differences with the above results might be ascribed to differences in culture media and culture conditions, and in the route of _C. parvum_ injection, which was intravenous in their experiments.

The results described here, and those of other workers mentioned above, demonstrated that cell-to-cell contact was not necessary for the co-operation between immune lymphoid cells and macrophages leading to activation of cytotoxicity in the macrophages. In addition, the results negated a possible criticism of the preceding experiments that spleen cells persisting in the microculture wells might be the cytotoxic effector cells.

## 6. AN IMMUNOLOGICALLY SPECIFIC COMPONENT OF THE IN VITRO ACTIVATION OF MACROPHAGES

An immunologically specific component of the _in vitro_ activation system was demonstrated by replacing _C. parvum_ strain CN6134 _in vitro_ with various other bacteria and conversely, by attempting to stimulate macrophages in the presence of _C. parvum_ by lymphocytes from mice treated with other adjuvants known to stimulate the reticulo-

endothelial system.

### 6.1 Comparison of Two Other Anaerobic Coryneform Bacteria with C. Parvum CN6134

In vitro activation was attempted using washed preparations of *C. parvum* strain NCTC 10390 and *Propionibacterium freudenreichii* NCTC 10470 in place of *C. parvum* CN6134. Table 17 illustrates that some anti-tumour cytotoxicity was obtained with strain 10390 but not with strain 10470. This result was not unexpected in view of the reported serological and other biological properties, including anti-tumour activity, of these bacteria (McBride *et al*., 1975a). Strains 10390 and 6134 exhibited antigen cross-reactivity, induced splenomegaly and had similar degress of anti-tumour activity *in vivo* and *in vitro*. By contrast, strain 10470 showed no cross-reactivity with the two *C. parvum* organisms and caused little splenomegaly or anti-tumour activity.

### 6.2 In vitro macrophage activation: Comparison of spleen cells from mice treated with various adjuvants

Mice were injected i.p. on day -11 with one of the following adjuvants; *C. parvum* CN6134, BCG (Glaxo), *Bordetella pertussis*, Freund's complete, Freund's incomplete. Spleen cells were tested in the activation assay in the presence of BCG, *B. pertussis* or *C. parvum*. Results have been summarised in Table 18.

Using spleen cells from *C. parvum*-treated mice, *B. pertussis* was able to mediate activation to some extent, but BCG was ineffectual.

TABLE 17 IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES: EFFECT OF REPLACING C.PARVUM (STRAIN 6134) IN THE IN VITRO TEST WITH OTHER STRAINS OF ANAEROBIC CORYNEFORM BACTERIA

| *In Vitro* Test Group | Bacterial Strain Used *In Vitro* | Spleen Cell Donor Treatment: None | Spleen Cell Donor Treatment: *C.parvum* (6134) Day -8 | C.I. | $P^{(a)}$ |
|---|---|---|---|---|---|
| Spleen Cells + Macrophages | None | 17,254(16,420-18,130) | 11,285(10,188-12,500) | 35 | <0.01 |
| | 6134 | 17,845(16,927-18,813) | 177(169-185) | 99 | ≪0.001 |
| | 10390 | 17,910(16,934-18,941) | 4,849(4,194-5,606) | 73 | <0.001 |
| | 10470 | 16,204(15,135-17,349) | 11,510(11,094-11,941) | 29 | <0.005 |
| Spleen Cells | None | 7,125(5,990-8,475) | 10,186(9,134-11,358) | Neg | <0.05 |
| | 6134 | 7,396(6,485-8,435) | 2,827(2,055-3,888) | 62 | |
| | 10390 | 11,953(8,839-16,163) | 16,516(15,899-17,157) | Neg | |
| | 10470 | 9,185(7,109-11,867) | 14,814(12,824-17,113) | Neg | |
| Additional Controls | Macrophages + 6134 | 17,266(16,696-17,855) | | | |
| | Tumour Alone | 10,900(10,178-11,673) | | | |

(a) Comparison of spleen cells from normal mice and *C.parvum*-treated mice.

It is not known if this reflects an antigenic relationship between *C. parvum* and *B. pertussis*. No cytotoxicity resulted from using *C. parvum* 6134 and spleen cells from animals treated with the other adjuvants. Negative results were also obtained in the BCG and *B. pertussis* groups in which the particular immunising organism was also present *in vitro*. Some activation might have been expected in these two groups in view of their known macrophage stimulating and anti-tumour properties (BCG: Evans and Alexander, 1972a; Alexander, 1973; Cleveland, Meltzer and Zbar, 1974; Hawrylko, 1975. *B. pertussis*: Malkiel and Hargis, 1961; Guyer and Crowther, 1969).

Data from the two experiments above demonstrate an immunologically specific component of activation: the antigen used *in vivo* to stimulate spleen lymphocytes must also be present *in vitro*. This confirms Christie and Bomford's findings using a similar experimental protocol (Christie and Bomford, 1975).

## 6.3 In vitro activation of macrophages: effect of using tumour-bearer spleen cells

The reasons why *C. parvum* should exhibit such potent anti-tumour properties are not known. One explanation that has been mooted is that *C. parvum* and tumour cells have shared antigens, a relationship that is known to exist with respect to BCG and hepatocarcinoma in guinea pigs (Bucana and Hanna, 1974; Minden, McClatchy, Wainberg and Weiss, 1974) and malignant melanoma in man (Minden, Sharpton and McClatchy, 1976). There is now evidence pointing to a similar phenomenon with *C. parvum*. James *et al*. (1976, 1977) have shown that *C. parvum* administration to normal and tumour-bearing mice

TABLE 18 IN VITRO ACTIVATION OF MACROPHAGE ANTI-TUMOUR CYTOTOXICITY BY C.PARVUM AND SPLEEN CELLS FROM C.PARVUM-TREATED MICE: EFFECT OF REPLACING C.PARVUM BY OTHER ADJUVANTS, BOTH IN THE PRE-TREATMENT OF SPLEEN CELL DONORS AND IN VITRO

| Adjuvant Treatment | | No. of Group | In Vitro Test Group: Macrophage + Spleen + Adjuvant | (P) Comparison with: | |
|---|---|---|---|---|---|
| In Vivo, ip Day -11 | In Vitro [a] | | | Group 1 | Group 2 |
| None | C.parvum | 1 | 5,780(4,728-7,067) | | <0.01 |
| 1.4mg C.parvum | C.parvum | 2 | 1,410(1,180-1,685) | <0.01 [b] | |
| | BCG | 3 | 3,975(3,732-4,234) | N.S. | <0.005 |
| | B.pertussis | 4 | 3,323(3,005-3,674) | <0.05 | <0.01 |
| 1.8 x $10^6$ organisms BCG | C.parvum | 5 | 5,614(5,145-6,126) | N.S. | <0.001 |
| | BCG | 6 | 4,901(4,473-5,369) | N.S. | <0.001 |
| 8 x $10^9$ organisms B.pertussis | C.parvum | 7 | 5,646(5,296-6,020) | N.S. | <0.001 |
| | B.pertussis | 8 | 4,601(4,341-4,876) | N.S. | <0.001 |
| 0.1ml Freund's Adjuvant — Complete | C.parvum | 9 | 5,432(5,060-5,831) | N.S. | <0.001 |
| 0.1ml Freund's Adjuvant — Incomplete | C.parvum | 10 | 5,888(5,574-6,219) | N.S. | <0.001 |

| Additional Controls | In Vitro adjuvant treatment of macrophages | | | |
|---|---|---|---|---|
| | None | C.parvum | BCG | B.pertussis |
| | 5,695(5,322-6,094) | 5,796(5,096-6,592) | 5,490(5,139-5,864) | 4,255(3,811-4,750) |

(a) The quantities of adjuvant bacteria used in vitro per well were: C.parvum, 0.7 µg; BCG, 9 x $10^2$ organisms; B.pertussis, 4 x $10^6$ organisms

(b) P values greater than 0.05 were considered not significant (N.S.)

results in the production of immunoglobulin of all classes and subclasses which will bind *in vitro* to tumour cells, syngeneic embryonic fibroblasts and syngeneic kidney and spleen cells. However, the significance of this is not clear in terms of *C. parvum*'s *in vivo* anti-tumour activity since tumour cells coated with such Ig did not exhibit reduced growth following s.c. or i.v. transplantation. These results suggested the existence of cross-reacting antigens on *C. parvum* and mouse tissue or tumour cells. Another explanation, not mutually exclusive to this, is provided by the finding that increased or *de novo* synthesis of autoantibodies occurs following *C. parvum* administration (McCracken, McBride and Weir, 1971; Cox and Keast, 1974; James, Clunie, Woodruff *et al.*, 1975).

In view of these results an experiment was set up in which spleen cells from tumour-bearing mice were used in the activation assay. Antigen cross-reactivity between *C. parvum* and tumour cells might be expected to result in activation of cytotoxicity. Three mice were injected subcutaneously with 1 x $10^5$ viable cultured tumour cells and their spleens were harvested 28 days later when the tumours averaged 18mm in diameter. Results indicated that there was a slight but significant increase in cytotoxicity using spleen cells from tumour-bearing mice (table 19). However, the presence of *C. parvum in vitro* had no effect on the level of cytotoxicity which implied that there was no evidence in this experiment of cross-reactivation between tumour cells and *C. parvum*.

ivity

TABLE 19 SLIGHT IN VITRO ACTIVATION OF ANTI-TUMOUR CYTOTOXICITY IN MACROPHAGES BY SPLEEN CELLS FROM TUMOUR-BEARING MICE

| In Vitro Test Group | Spleen Cell Donors | | C.I. | P[b] |
|---|---|---|---|---|
| | Normal Mice | Tumour-Bearing Mice[a] | | |
| Spleen + Macrophage + C.parvum | 18,526(17,968-19,101) | 14,498(14,056-14,953) | 22 | $<0.001$[c] |
| Spleen + Macrophage | 16,669(16,059-17,302) | 13,197(12,536-13,894) | 21 | $<0.01$[d] |
| Spleen + C.parvum | 9,138(8,167-10,225) | 10,976(10,239-11,765) | Neg | |
| Spleen | 9,853(9,380-10,349) | 8,946(7,991-10,016) | 9 | N.S. |
| Macrophage + C.parvum | 18,857(18,169-19,570) | | | |
| Tumour Alone | 13,947(12,547-15,503) | | | |

(a) Mice injected subcutaneously in left thigh with $1 \times 10^5$ viable cultured tumour cells suspended in 100 µl PBS on day -28.

(b) Comparison of effects of spleens from normal mice with spleens from tumour-bearing mice. P values greater than 0.05 were considered not significant.

(c) Although there are significant differences between normal spleens and tumour-bearing spleens, the presence of C.parvum is not contributory to anti-tumour cytotoxicity (compare line 1 with line 2). Thus, this experiment does not support the hypothesis that C.parvum's anti-tumour properties may be due to antigens shared between C.parvum and tumour cells.

# R E S U L T S - P A R T 2

## *IN VITRO* BEHAVIOUR OF *C. PARVUM*-ACTIVATED CYTOTOXIC MACROPHAGES

IN VITRO BEHAVIOUR OF C. PARVUM-ACTIVATED CYTOTOXIC MACROPHAGES

It was observed that macrophages activated with C. parvum in vivo quickly lost their anti-tumour cytotoxicity during in vitro cultivation. This loss of activity, generally complete by 24 hours, was measured by incubating macrophages for various periods before the addition of tumour cells. $^{125}$IUDR was added to culture wells 24 hours later to assess cytotoxicity. Typical results have been summarised in Table 20.

It was further noticed that this loss of anti-tumour cytotoxicity could be delayed by cultivating the macrophages with C. parvum (Table 21). In these experiments C. parvum was added to macrophage monolayers immediately following the washing procedure to remove non-adherent PE cells, and, at various times later, tumour cells were added. High macrophage cytotoxicity was sustained by the presence of C. parvum for the first 24 hours of culture but by 48 hours anti-tumour activity was again declining (Experiment 2, Table 21). In some experiments it was sometimes possible to regenerate lost cytotoxicity after 24 hours by the addition of C. parvum along with tumour cell targets (line 4, Experiment 1; Table 21). However, this phenomenon of reactivation following prolonged culture was not observed in all experiments (e.g. line 4, Experiment 2), and never following culture in excess of 30 hours (Experiments 1 and 2, Table 21).

Added C. parvum thus seems to provide a continuing stimulation maintaining anti-tumour activity. This effect has been summarised

TABLE 20 ANTI-TUMOUR CYTOTOXICITY OF MACROPHAGES FROM C. PARVUM-TREATED MICE.[a] LOSS OF CYTOTOXICITY FOLLOWING IN VITRO CULTURE

| Period of Macrophage Culture (hr) (b) | Effector to Target Ratio | Counts per Minute in Cultures with Macrophages from (c) | | | |
|---|---|---|---|---|---|
| | | Normal mice | C. parvum-Treated Mice | C.I. | P (d) |
| 3 | 40 | 18,145 | 195 | 99 | <0.001 |
| | 20 | 18,348 | 428 | 98 | <0.001 |
| | 10 | 14,071 | 2,637 | 81 | <0.001 |
| 18 | 40 | 12,368 | 1,919 | 84 | <0.001 |
| | 20 | 13,250 | 5,601 | 58 | <0.001 |
| | 10 | 11,682 | 19,693 | Neg | - |
| 24 | 20 | 20,709 | 23,138 | Neg | - |
| | 20 | 16,829 | 19,109 | Neg | - |
| | 20 | 14,429 | 13,163 | 9 | N.S. |
| 48 | 40 | 26,973 | 35,583 | Neg | - |
| | 20 | 22,931 | 27,394 | Neg | - |
| | 10 | 20,574 | 26,890 | Neg | - |

(a) 0.2ml C. parvum i.p. 10 days previously

(b) Time of incubation of macrophages in vitro before the addition of tumour cells

(c) Geometric mean of 5 cultures. Limits of 1 standard error have been omitted

(d) Comparison of C. parvum-treated group with normal group; P values greater than 0.05 were considered to be not significant (N.S.). Results have been summarised from 3 experiments.

TABLE 21 MAINTENANCE OF ANTI-TUMOUR CYTOTOXICITY IN CULTURES OF MACROPHAGES FROM C. PARVUM-TREATED[a] MICE BY THE ADDITION OF C. PARVUM IN VITRO

| Exp. No. | *In Vitro* Incubation (hr) Before Adding | | *In Vivo* Treatment | Counts per minute in Cultures | | C.S.I[c] | P[d] |
|---|---|---|---|---|---|---|---|
| | C.parvum[b] | Tumour | | Without C. parvum | With C. parvum | | |
| 1 | 1½ | 24[e] | None | 30,351(24,633-37,397) | 37,613(33,193-42,622) | Neg | |
| | | | C. parvum | 23,360(19,909-27,409) | 4,902(4,177-5,751) | 79 | <0.001 |
| | 30 | 30 | None | 22,204(20,153-24,463) | 21,237(20,429-22,077) | 4 | N.S. |
| | | | C. parvum | 27,538(26,654-28,452) | 13,452(12,279-14,736) | 51 | <0.001 |
| | 48 | 48 | None | 22,931(20,665-25,446) | 25,067(23,674-26,541) | Neg | |
| | | | C. parvum | 27,394(25,672-29,231) | 23,884(21,836-26,122) | 13 | N.S. |
| | 72 | 72 | None | 21,288(18,170-24,940) | 17,030(14,443-20,080) | 20 | N.S. |
| | | | C. parvum | 25,119(23,533-26,812) | 21,683(20,893-22,503) | 14 | N.S. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2 | 1½ | 24 | None | 12,553(10,821-14,562) | 10,792(9,256-12,584) | 14 | N.S. |
| | | | C. parvum | 23,224(21,494-25,093) | 3,145(2,781-3,556) | 86 | <0.001 |
| | 24 | 24 | None | 12,552(10,821-14,562) | 13,745(11,627-16,249) | Neg | |
| | | | C. parvum | 23,224(21,494-25,093) | 20,559(19,302-21,898) | 11 | N.S. |
| | 1½ | 48 | None | 17,761(16,276-19,381) | 15,188(13,525-17,055) | 14 | N.S. |
| | | | C. parvum | 35,531(32,649-38,647) | 26,474(24,777-28,287) | 25 | <0.025 |
| | 48 | 48 | None | 17,761(16,276-19,381) | 20,981(19,199-22,932) | Neg | |
| | | | C. parvum | 35,521(32,649-38,647) | 27,662(25,046-30,552) | 22 | N.S. |

(a) 1.4mg C. parvum injected i.p. 10 days previously

(b) P.E. cell monolayers washed once before addition of C. parvum

(c) C.S.I. = Cytotoxic Stimulation Index

(d) Comparison of groups incubated with or without C. parvum
P values greater than 0.05 were considered not significant (N.S.)

(e) In this group only, C. parvum was removed by two washes before the addition of tumour cells

in the tables as a cytotoxic stimulation index (CSI). (See Materials and Methods).

Loss of anti-tumour activity with in vitro culture and its prevention by the addition of C. parvum was also observed in in vitro activated macrophages. However, because of the ease with which they are obtained, only in vivo stimulated macrophages have been used in the following experiments, designed to assess the effects of various treatments on the restimulation phenomenon.

Effect of x-irradiation of macrophages

Peritoneal exudate cells from normal and C. parvum-treated mice were subjected to various doses of x-irradiation prior to seeding in microculture plates. 90 minutes later wells were washed to remove non-adherent cells, and C. parvum or growth medium added. 24 hours later wells were again washed to remove the bacteria and tumour cells added to assess cytotoxicity. The results (Table 22) clearly indicated that x-irradiation (up to 800 rads) did not interfere with the maintenance of cytotoxicity by in vitro C. parvum. This experiment fails to exclude the possible contribution to restimulation of activated T cells or plasma (B) cells which are known to be radioresistant (Anderson and Warner, 1976).

Effect of sodium aurothiomalate

The C. parvum-induced retention of cytotoxicity in cultures of in vivo-stimulated macrophages was markedly inhibited when sodium

TABLE 22 MAINTENANCE OF ANTI-TUMOUR CYTOTOXICITY IN CULTURES OF MACROPHAGES FROM C. PARVUM-TREATED MICE BY THE ADDITION OF C. PARVUM IN VITRO. THE EFFECT OF PRIOR X-IRRADIATION OF PE CELLS

| *In Vivo* Treatment | Radiation[a] Dose (Rads) | Counts Per Minute in Cultures | | C.S.I. | P[c] |
|---|---|---|---|---|---|
| | | Without *C. parvum* | With *C. parvum*[b] | | |
| None | None | 22,959(21,382-24,652) | 19,735(18,137-21,473) | 14 | N.S. |
| | 200 | 20,624(18,164-23,418) | 21,406(19,001-24,116) | Neg | |
| | 400 | 20,503(19,129-21,976) | 22,163(20,290-24,209) | Neg | |
| | 800 | 23,088(20,847-25,569) | 17,513(15,956-19,221) | 24 | N.S. |
| *C. parvum* ip Day -10 | None | 10,139(9,406-10,929) | 1,534(1,404-1,676) | 85 | <0.001 |
| | 200 | 13,426(12,234-14,735) | 3,055(2,207-4,229) | 77 | <0.005 |
| | 400 | 14,808(12,832-14,859) | 2,429(2,240-2,634) | 84 | <0.001 |
| | 800 | 19,084(16,460-22,127) | 3,461(2,889-4,147) | 82 | <0.001 |

(a) PE cells irradiated prior to seeding in culture plates

(b) $1\frac{1}{2}$ hours after plating, PE cell monolayers were washed once and *C. parvum* added; 24 hours later the wells were washed twice to remove *C. parvum* before the addition of tumour cells

(c) Comparison of groups incubated with or without *C. parvum*. P values greater than 0.05 were considered not significant (N.S.).

TABLE 23 MAINTENANCE OF ANTI-TUMOUR CYTOTOXICITY IN CULTURES OF MACROPHAGES FROM C. PARVUM-TREATED MICE BY THE ADDITION OF C. PARVUM IN VITRO. EFFECT OF THE PRESENCE OF GOLD SALT IN THE CULTURES

| In Vivo Treatment | Gold Salt In Vitro (μg/well) | Counts Per Minute in Cultures | | C.S.I. | P [a] |
|---|---|---|---|---|---|
| | | Without C. Parvum | With C. Parvum | | |
| None | None | 20,709(18,074-23,727) | 26,820(15,202-18,542) | Neg | |
| | 10 | 22,699(21,271-24,223) | 18,188(17,125-19,317) | 20 | $<0.05$ |
| | 20 | 21,976(21,110-22,878) | 19,283(17,699-21,009) | 12 | N.S. |
| C. parvum ip Day -7 | None | 23,138(22,292-24,017) | 1,024(912-1,150) | 96 | $\ll 0.001$ |
| | 10 | 26,289(25,315-27,301) | 20,644(19,766-21,562) | 21 | $<0.01$ |
| | 20 | 24,910(23,873-25,993) | 26,183(24,996-27,425) | Neg | |

(a) Comparison of groups incubated with or without C. parvum. P values greater than 0.05 were considered not significant (N.S.).

aurothiomalate was added to the cultures (Table 23). This result parallels the effects of gold salt on the *in vitro* activation system (*vide supra*, Part 1.).

Effect of T cell deprivation of macrophage donor

The T cell dependency of *C. parvum*-mediated maintenance of cytotoxicity in cultured macrophages was investigated using macrophages from *C. parvum*-treated B mice (thymectomised, irradiated, bone-marrow reconstituted mice). It was apparent from this experiment that cytotoxicity was not fully retained, in the presence of *C. parvum*, in macrophages harvested 3 or 6 days post-*C.parvum* injection, regardless of thymic function. Restimulation was only apparent in macrophages obtained 12 days post-*C. parvum* from immunologically intact mice. The equivalent thymectomised group did not have macrophages which could be restimulated. It would thus appear that the phenomenon is a T cell dependent event. This might also explain the delay in the appearance of the capacity for restimulation in macrophages from intact mice - the T cell reaction to *C. parvum* is perhaps not fully developed until after the sixth day post-injection. This is also similar to the delay of about 6 days in the appearance of lymphocytes capable of activating macrophages *in vitro*.

Specificity of restimulation

To determine whether the maintenance of cytotoxicity in activated macrophages by *C. parvum* was immunologically specific, two other

TABLE 24 MAINTENANCE OF ANTI-TUMOUR CYTOTOXICITY IN CULTURES OF MACROPHAGES FROM C. PARVUM-TREATED MICE BY THE ADDITION OF C. PARVUM IN VITRO. EFFECT OF T CELL DEPRIVATION[a] OF MACROPHAGE DONOR

| In Vivo Treatment | | Counts Per Minute In Cultures | | C.S.I. | P[b] |
|---|---|---|---|---|---|
| T Cell Deprivation | *C. parvum* ip | Without *C. Parvum* | With *C. Parvum* | | |
| None | None | 14,429(13,323-15,628) | 13,633(12,629-14,716) | 6 | N.S. |
| | Day -3 | 15,291(14,349-16,294) | 9,413(8,562-10,348) | 38 | <0.005 |
| | Day -6 | 10,524(9,518-11,636) | 6,899(6,471-7,356) | 34 | <0.01 |
| | Day -12 | 13,163(12,123-14,292) | 3,246(3,080-3,420) | 75 | <0.001 |
| T Cell Deprivation | None | 10,054(8,475-11,927) | 13,123(12,186-14,133) | Neg | |
| | Day -3 | 10,128(9,237-11,105) | 6,204(5,656-6,804) | 39 | <0.01 |
| | Day -6 | 7,326(6,469-8,297) | 5,048(4,556-5,593) | 31 | <0.05 |
| | Day -12 | 9,906(9,103-10,781) | 8,133(7,673-8,620) | 18 | N.S. |
| Tumour Control | | 8,815(7,182-10,820) | 12,577(10,438-15,154) | Neg | |

(a) See Materials and Methods for details of this procedure

(b) Comparison of groups incubated with or without *C. parvum*. P values greater than 0.05 were considered not significant (N.S.)

anaerobic coryneform bacteria, C. parvum NCTC 10390 and Propionibacterium freudenreichii NCTC 10470, were compared with C. parvum CN6134 in the test system. Table 25 shows that a restimulation effect could be obtained with all bacteria tested but that the degree of cytotoxicity depended on the strain. The antigenically related strains CN6134 and NCTC 10390 (McBride et al., 1975a) gave maximal cytotoxicity and P. freudenreichii was much less effective. The restimulation effect is thus partially specific. (See also the results using these organisms in the in vitro activation assay in Part 1).

Summary of experiments on the maintenance of cytotoxicity of cultured C. parvum-activated macrophages

In conclusion, the maintenance, by C. parvum, of the cytotoxicity of cultured, activated macrophages has been shown to be radioresistant, sensitive to gold salt, and at least partially specific. In addition, the results using 'B' mice suggested that T cells were involved but whether in vitro or in vivo or both, was not clear. However, the fact that activated, cytotoxic macrophages could be recovered from C. parvum-treated 'B' mice as well as from thymically intact mice (see table 13), would suggest that the failure to restimulate 'B' mouse macrophages reflected a lack of appropriately stimulated peritoneal T cells in the culture wells rather than some in vivo T cell-dependent alteration of macrophage function. The results of the irradiation experiment require that these T cells be radioresistant. It would be interesting to see if anti-θ treatment of PEC monolayers had any effect on restimulation. There is no evidence of

TABLE 25 MAINTENANCE OF ANTI-TUMOUR CYTOTOXICITY IN CULTURES OF MACROPHAGES FROM C. PARVUM-TREATED MICE BY THE ADDITION OF C. PARVUM IN VITRO. EFFECT OF SUBSTITUTING IN VITRO C. PARVUM WITH OTHER BACTERIAL STRAINS

| *In Vivo* Treatment | Bacterial Strain Added *in Vitro*[a] | Counts Per Minute | C.S.I. | P[b] |
|---|---|---|---|---|
| None | None | 16,829(16,187-17,497) | | |
| | 6134 | 17,266(16,696-17,855) | Neg | |
| | 10390 | 18,086(16,818-19,449) | Neg | |
| | 10470 | 17,309(16,402-18,267) | Neg | |
| *C. parvum* (6134) ip Day -10 | None | 19,109(18,032-20,251) | | |
| | 6134 | 648(611-687) | 97 | $<0.001$ |
| | 10390 | 1,223(1,149-1,302) | 94 | $<0.001$ |
| | 10470 | 5,865(5,485-6,270) | 69 | $<0.001$ |

(a) 0.70µg strain 6134 per well and the equivalent quantity for 10390 and 10470

(b) Comparison with line 5

B cell involvement in the process but this could be resolved by using an anti-B cell serum. It has been postulated (Ghaffar and Cullen, 1976) that the loss of cytotoxicity with culture might be due to the loss or degradation of macrophage-bound macrophage activating factor (MAF) and that the addition of C. parvum to culture wells stimulates the regeneration of MAF by peritoneal lymphocytes.

Hibbs (1975b) came to a similar conclusion using BCG-activated macrophages which, he found, lost their tumoricidal potential when cultured for 48-72 hours. Cytotoxicity could be retained, however, if specifically sensitised peritoneal lymphocytes and tuberculin protein were added to the macrophage cultures. Hibbs also reported that macrophages occasionally retained some cytotoxicity when tuberculin protein alone was added to cultures, a finding which mirrors the C. parvum results described above.

Some of the results presented in Part 2 have been published previously (Ghaffar and Cullen, 1976; Ghaffar et al., 1976b).

# DISCUSSION

## DISCUSSION

This thesis has attempted to elucidate the mechanisms underlying the anti-tumour effects of C. parvum particularly with respect to the production of cytotoxic macrophages, believed to be important anti-tumour effector cells. Two approaches were made; a direct one attempting to stimulate non-cytotoxic macrophages with C. parvum and/or with serum containing C. parvum antibodies, and secondly an indirect one using lymphocytes, or their mediators, from C. parvum-treated animals. The latter approach, only, was to give macrophages with in vitro anti-tumour activity. As the work developed it became clear that in addition to this mechanism involving lymphocyte mediation, there must also be another, unknown, mechanism operating in vivo to account for the early appearance of cytotoxic macrophages following C. parvum administration. It was also observed that cytotoxic macrophages rapidly lost the ability to inhibit the growth of tumour cells when cultured in vitro but that this loss of activity could be delayed by adding C. parvum to the macrophage cultures. This implied that continuous stimulation of macrophages was required in vivo to explain the prolonged anti-tumour effects of a single C. parvum injection. These mechanisms are discussed below in relation to the literature on the anti-tumour and other biological effects of C. parvum.

### 1. MACROPHAGES AS ANTI-TUMOUR EFFECTOR CELLS

1.1 Throughout this work the anti-tumour effector cell has been assumed to be the macrophage. This assumption was based largely on the observation that the predominant cell type in the adherent peri-

toneal cell monolayers had the characteristics of the macrophage, as determined by morphology under phase contrast microscopy, staining properties, phagocytosis of C. parvum, and trypsin-resistant adherence to glass and plastic.

Following incubation with C. parvum and lymphoid cells from C. parvum-treated mice peritoneal macrophages exhibited the morphological characteristics of activated macrophages as described by others of spreading amoeboid appearance with pseudopodia and ruffling membrane, and containing many phase-dense lysosomes and phase-lucent vacuoles (Cohn, 1968; Fedorko and Hirsch, 1970; Adams, Biesecker and Koss, 1973). Further evidence that such activated macrophages were responsible for the *in vitro* anti-tumour effect, was shown by the sensitivity of cytotoxicity to the addition of sodium aurothiomalate to the culture wells, either during peritoneal cell adherence, or during the activation process itself (*vide infra*). In addition, the ineffectiveness of prior x-irradiation of peritoneal cells to affect cytotoxicity also lends support to this macrophage evidence (*vide infra*).

## 1.2 Nature of the anti-tumour effect of C. parvum-activated macrophages

Macrophages from mice treated with reticuloendothelial stimulants such as BCG and C. parvum are generally regarded as being non-specifically cytotoxic. Peritoneal exudate cells and spleen cells from mice treated i.p. with C. parvum have been shown to inhibit the *in vitro* growth of methylcholanthrene-induced fibrosarcomas of both CBA and A/Hej mice and also of murine embryonic fibroblasts (Cullen

and Ghaffar - unpublished observations). This non-specific cytotoxicity has also been reported by others (Jones, McBride and Weir, 1975). However, the question of an antigenic relationship between C. parvum and tumour cells or normal murine tissue, throws some doubt on whether these results truly indicate a non-specific effect (see discussion on specificity below).

Although the design of the anti-tumour assay used in this study was such that it could not distinguish between growth inhibition and killing of tumour cells, observation of the culture wells using trypan blue and phase contrast microscopy showed that death of target cells did occur in many experiments.

By harvesting tumour cells with trypsin following incubation on monolayers of cytotoxic macrophages and then transferring them to a fresh plate for further culture, it was possible to show that both cytostasis and killing of tumour cells was occurring. Results from an experiment of this type are given on the next page (Table 26). In this case tumour cells had been added to C. parvum-activated peritoneal macrophages in a Petri dish, and 48 hours later adherent and non-adherent tumour cells were harvested with the aid of trypsin and washed in growth medium. Viable cell counts were made using the trypan blue exclusion test (see appendix for details). Tumour cells were then replated in fresh Petri dishes. After a further 72 hours the total cell content (adherent and non-adherent) of each dish was harvested and viable counts again performed.

It can be seen that in the untreated control and the unactivated macrophage control plates the total number of viable cells increased with time, whereas, in comparison, the tumour cell population exposed

TABLE 26 FAILURE OF TUMOUR CELLS EXPOSED TO ACTIVATED MACROPHAGES TO PROLIFERATE IN VITRO EVEN WHEN REMOVED FROM THE PRESENCE OF SUCH MACROPHAGES: DEMONSTRATION OF CYTOTOXIC AND CYTOSTATIC COMPONENTS IN THE ANTI-TUMOUR EFFECT OF ACTIVATED MACROPHAGES

| Initial Treatment of Tumour Cells | Number of Trypsinised Cells per Dish[a] ($\times 10^{-4}$) | | | | | |
|---|---|---|---|---|---|---|
| | After 2 Days[b] | | | 3 Days after Transfer | | |
| | Viable[c] | Dead | % Dead | Viable | Dead | % Dead |
| None | 8.25 | 0.28 | 3.4 | 51.30 | 4.50 | 8.8 |
| Normal Macrophages | 8.80 | 0.33 | 3.7 | 84.30 | 3.40 | 4.0 |
| Activated Macrophages[d] | 5.00 | 0.60 | 12.0 | 5.60 | 1.20 | 21.4 |

(a) This experiment was performed in 30 mm plastic Petri dishes. Figures given are for the total numbers of cells per dish, non-adherent (i.e. free in the culture supernatant) as well as trypsin-sensitive adherent cells.

(b) After 48 hours tumour cells were harvested with 0.25% trypsin, washed and counted. The tumour cells from each dish were then transferred *in toto* to a fresh dish and incubated for a further 72 hours.

(c) Viability was assessed by the trypan blue dye exclusion test.

(d) Macrophages were activated in the usual way: macrophage monolayers exposed to *C.parvum* and spleen cells for 24 hours and then washed three times. $2.5 \times 10^6$ peritoneal exudate cells were seeded initially in the dishes and the numbers of all other cells used were in proportion to this to give the same cell ratios that would be used in a 96-well culture plate. $12.5 \times 10^4$ tumour cells were added initially to each plate.

to activated macrophages was virtually static. The percentage of dead cells from the activated group was also considerably higher.

Experiments of this type have several failings. For example, it is impossible to assess the number of cells that may have lysed during the incubation periods. Another criticism is that cytotoxic macrophages could have been carried over from the first harvest to the second incubation. However, this would be unlikely to affect growth of tumour cells because the numbers of macrophages involved would be small and they would probably have lost their cytotoxicity after the initial 48 hours of culture. (see Results - Part 2). The experiment does show, nevertheless, that tumour cells exposed to activated macrophages fail to recover (at least within 72 hours) even when removed from those macrophages. The increase in the percentage of dead cells with time also suggests that some tumour cells experienced an initial cytostasis, followed at a later time by death.

This experiment also illustrates an observation, frequently made, that normal macrophages can promote tumour growth *in vitro*. This has also been noted by other workers (Keller, 1976a). The sinister possibility must be considered that the macrophage content of many tumours may in fact be stimulating tumour growth within the tumour mass. Prehn (1972, 1976) has argued that the immune system may actually promote tumour growth. Such a phenomenon may account in part for the anti-tumour action of *C. parvum* since one of its effects is T cell suppression thereby blocking any immunostimulation of tumour growth.

The actual mechanism by which *C. parvum*-activated macrophages exert their *in vitro* cytostatic or cytotoxic effects is not known for

certain. From the work of others several possibilities exist. Bennet, Old and Boyse (1964) described a system in which target tumour cells were phagocytosed and killed intracellularly provided that an opsonic antibody were present. Extracellular killing by labile, lytic factors released into the culture medium have also been described (Kramer and Granger, 1972; Currie and Basham, 1975; Melsom, Sanner and Seljelid, 1975; Sethi and Brandis, 1975). These factors could be complement cleavage products such as C3a, which has also been shown to be cytolytic (Ferluga, Schorlemmer, Baptista and Allison, 1976). A third system requires macrophage-target cell contact (Granger and Weiser, 1964; Evans and Alexander, 1970; Hibbs, 1974). Hibbs (1974) was able to demonstrate that this type of macrophage-mediated killing occurred by the transfer of lysosomes to the cytoplasm of target cells which subsequently underwent heterolysis. It is likely that C. parvum-activated macrophages also exert their anti-tumour effects in this way since these effects can be abrogated by gold salt (tables 8 and 9) which is known to inhibit lysosome hydrolases (Persellin and Ziff, 1966; Ennis et al., 1968). Administration of C. parvum leads to an increase in macrophage lysosomal enzymes (McBride, Jones and Weir, 1974).

### 1.3 The retention of macrophage cytotoxicity in cultures by C. parvum

As described in Results - Part 2, activated macrophages lose their anti-tumour cytotoxicity within the first 24 hours of culture, a loss which has also been observed by others in a lymphoma system (Evans and Alexander, 1970) and with BCG-activated macrophages (Hibbs,

1975b). The addition of C. parvum to the macrophage cultures delayed this loss of activity, or even regenerated it in some experiments. Analysis of this effect showed the process to be T cell dependent, radioresistant and at least partially specific. Since cytotoxic macrophages could be recovered from both T cell-deprived mice and thymically intact mice (Ghaffar et al., 1975), the inability of cytotoxic macrophages from 'B' mice to respond to added C. parvum in culture implied that the in vitro restimulation process required T cells, rather than that the macrophages from 'B' mice were functionally different from those of thymically intact mice. It is not yet known whether B cells are involved in the phenomenon of restimulation.

It has been postulated (Ghaffar and Cullen, 1976) that the loss of cytotoxicity with culture might be due to the loss or degradation of macrophage-bound macrophage activating factor (MAF) and that the addition of C. parvum to culture wells stimulates the regeneration of MAF by the few peritoneal lymphocytes persisting in the wells. In this respect it is interesting that Hibbs (1975b) using cytotoxic macrophages from BCG-infected mice could cause the retention of cytotoxicity in culture by the addition of tuberculin protein and sensitised lymphocytes, the addition of either agent alone being without effect.

Possibly of some relevance to these observations are the recent immunological studies using anti-alpha-2-macroglobulin sera showing that peritoneal macrophages from C. parvum-treated mice exhibit a different pattern of intracellular staining to those from untreated mice, and that most of this staining is lost within 16 hours of

culture (T.A. Varley - personal communication). Alpha-2-macroglobulin may be a carrier for MAF (McDaniel, Laudico and Papermaster, 1976; see also section below on activation of macrophages by lymphocyte culture supernatants).

## 2. IN VITRO ACTIVATION OF MACROPHAGES BY SPLEEN AND LYMPH NODE CELLS

It was found that spleen cells obtained from mice injected with C. parvum, either intraperitoneally (i.p.) between 6 and 21 days before, or intravenously (i.v.) between 6 and 14 days before, could stimulate anti-tumour cytotoxicity in normal macrophages, but only in the presence of C. parvum. The effect was never present until after day 6 (data not presented) despite the fact that spleen and peritoneal exudate cells from mice given C. parvum systemically, contain a population of cytotoxic cells by day 4 (Ghaffar et al., 1974) or even by day 2 (Jones, McBride and Weir, 1975). This discrepancy suggests that in vivo macrophage activation occurs very rapidly following C. parvum injection and by a mechanism other than that involving sensitised lymphocytes. It could be argued that with the i.p. route macrophages are being activated by peritoneal lymphocytes in the early stages, although the lack of activation before day 6 with the intravenous route makes this less likely. Possible mechanisms accounting for the early activation of anti-tumour cytotoxicity are discussed later.

Subcutaneously administered C. parvum was largely ineffective in producing spleen cells capable of in vitro activation.

These results parallel remarkably closely the development of

cytotoxic peritoneal exudate cells in C. parvum-treated mice as assessed by *in vitro* tests (Ghaffar and Cullen, 1977).

Peripheral lymph node cells from i.p. treated mice could also activate macrophages but to only a limited extent, and only then if the lymphocyte to macrophage ratio was increased four-fold. Cells from nodes draining the site of a subcutaneous C. parvum injection were the most effective, but again it required four times the number of cells than would have been the case using spleen cells. The effect of i.v. injection of C. parvum on the macrophage-activating capacity of lymph node cells has not yet been investigated.

This variation in expression of anti-tumour effects between the routes of C. parvum administration has been demonstrated repeatedly in *in vivo* studies. Both the i.p. and the i.v. routes are generally regarded as being effective therapeutically (Scott, 1974c; Milas *et al*., 1974a; Woodruff and Dunbar, 1975), whereas the subcutaneous route is only of value when the injection is at, or close to, the tumour site (Scott, 1974b; Likhite and Halpern, 1974; Woodruff and Dunbar, 1975). In addition, C. parvum given by the s.c. route has little effect on circulating immunoglobulin levels including antibodies to C. parvum which is in marked contrast to the increases in mice treated by the i.p. route (Woodruff, McBride and Dunbar, 1974; James *et al*., 1976).

## 3. SPECIFICITY OF THE IN VITRO MACROPHAGE ACTIVATION

The immunologically specific nature of the *in vitro* activation was demonstrated by replacing C. parvum 6134 with other bacterial adjuvants both *in vitro* and *in vivo*. The use of Glaxo strain BCG

and P. freudenreichii 10470 in vitro with C. parvum-immune lymphocytes was completely ineffective. However, C. parvum 10390 and, to a lesser extent, B. pertussis did mediate some activation. 10390 is antigenically related to 6134 and, in addition, exhibits similar reticuloendothelial stimulating and anti-tumour properties (McBride et al., 1975a). It is not known whether C. parvum 6134 cross-reacts with B. pertussis. These results, therefore, strongly indicate an immunologically specific component in in vitro macrophage activation. An alternative explanation might be that the activation obtained with 10390 and B. pertussis was due simply to the biologically active nature of these bacteria. This is unlikely since 10390 was not as effective as 6134 in the assay although both strains appear to have similar anti-tumour properties (McBride et al., 1975a). The conclusion that in vitro macrophage activation is an immunologically specific phenomenon is supported by the work of Christie and Bomford (1975) using a similar protocol.

Attempts to activate macrophages with BCG or B. pertussis and spleen cells from mice previously injected with these organisms were unsuccessful although both agents have known anti-tumour properties (Evans and Alexander, 1972a; Hawrylko, 1975; Guyer and Crowther, 1969). James, Wilmott, Milne and Cullen (1977) using the same B. pertussis and BCG preparations used in this study found that B. pertussis had some slight in vivo anti-tumour activity but that the BCG preparation had none. The inactivity of the Glaxo BCG strain used here may reflect its reported lower virulence and lack of persistence in mice as compared to other strains (Evans and Alexander, 1976). Fortner, Hanna and Coggin (1974) compared the Glaxo BCG with the Phipps strain

of BCG in guinea pigs, and found significant differences in terms of growth kinetics and in anti-tumour activity.

It has been postulated that C. parvum's anti-tumour activity might be due to the stimulation of a specific anti-tumour immune response, either directly through cross-reacting antigens on tumour cells and C. parvum, or merely by close association of tumour and C. parvum in vivo. Evidence for this is discussed below.

Sharing of antigenic determinants has been demonstrated between BCG and hepatocarcinoma cells in guinea pigs (Borsos and Rapp, 1973; Minden, McClatchy, Wainberg and Weiss, 1974; Bucana and Hanna, 1974) and recently between BCG and human malignant melanoma cells (Minden, Sharpton, and McClatchy, 1976). The existence of a similar antigenic relationship between C. parvum and tumour cells might explain the observations of James and his colleagues (1976, 1977) that administration of adjuvant-like bacteria such as C. parvum, P. freudenreichii and B. pertussis in the mouse resulted in a significant increase in certain serum immunoglobulins which could bind in vitro to both tumour cells and normal mouse cells. However, other equally plausible explanations might be: a) an increased or de novo synthesis of autoantibodies (McCracken, et al., 1971; Cox and Keast, 1974; James et al., 1975); b) an increase in preexisting anti-tumour antibodies (Martin and Martin, 1974); c) the production of unique anti-tumour antibodies. A fourth possibility that the results were due to binding of cytophilic Ig via Fc receptors was excluded on two counts; firstly, that the cultured tumour cells were virtually devoid of Fc receptors (James et al., 1976; Szymaniec and James, 1976) and secondly, that binding could not be blocked with aggregated IgG. The significance

of these _in vitro_ Ig-binding experiments was not clear since incubation of tumour cells in sera rich in these immunoglobulins did not affect their growth when subsequently transplanted.

The question of shared antigens between _C. parvum_ and fibrosarcoma cells was investigated using the _in vitro_ macrophage activation assay. Activation was attempted using spleen cells from mice bearing tumours of about 18mm diameter (table 19). Spleen cells from such mice have been shown to be cytotoxic to tumour cells _in vitro_ (Cullen - unpublished observations) and also to have elevated K cell cytotoxicity (Ghaffar, Calder and Irvine, 1976a).

Although some increase in macrophage anti-tumour activity was observed, it was unaffected by the presence of _C. parvum_. This experiment thus failed to support the hypothesis of shared antigenicity. It might be worthwhile to repeat this experiment using spleen cells from mice with varying sizes of tumour, or with the cells from lymph nodes draining the tumour site.

_C. parvum_-enhanced or _C. parvum_-initiated tumour-specific responses have been reported in two experimental systems, one in which _C. parvum_ was injected intratumour, and the other in which _C. parvum_ was administered together with irradiated tumour cells. Intratumour inoculation of _C. parvum_ can result in partial or complete regression of the tumour with consequent strong, specific anti-tumour immunity (Likhite and Halpern, 1974; Scott, 1974b). This effect is T cell-dependent (Scott, 1974b; Woodruff and Dunbar, 1975; Woodruff and Warner, 1977), and partly dependent on a specific immune response to _C. parvum_ (Scott, 1974b). Intratumour injection of BCG can also cause regression and seems to be associated with the development of a

delayed hypersensitivity reaction to the bacteria in the bed of the tumour as well as generation of a tumour specific response (Zbar, Bernstein and Rapp, 1971; Zbar, Bernstein, Bartlett _et al_., 1972; Hawrylko and Mackaness, 1973).

T cell-dependent augmentation of tumour-specific immunity has also been achieved in the mouse by injection of mixtures of _C. parvum_ and irradiated tumour cells subcutaneously into the footpad but not by intravenous or intra-peritoneal injections (Scott, 1975; Bomford, 1975; Tuttle and North, 1976a; Scott and Bomford, 1976). Injection of _C. parvum_ and irradiated tumour cells at separate sites also gave tumour immunity provided the sites had common lymphoid drainage (Scott, 1975). In contrast to the intratumour experiments this process was not dependent on the development of a specific immune response to _C. parvum_ (Scott, 1975).

It can be seen, therefore, that, depending on circumstances, _C. parvum_ administration can give rise to a non-specific anti-tumour effect or a specific anti-tumour response. It would be interesting to know if lymphoid cells from mice with a strong _C. parvum_-enhanced tumour-specific immunity could activate macrophages _in vitro_ and whether the presence of _C. parvum_ would be required. Scott (1975) demonstrated that such specific immunity could be transferred to normal animals by transfer of lymphocytes. Adoptive transfer of immunity in a similar model was shown to be mediated by short-lived T cells (Tuttle and North, 1976b).

## 4. CHARACTERISATION OF THE CELLS INVOLVED IN ACTIVATION

Various approaches utilising x-irradiation, anti-macrophage serum and sodium aurothiomalate were made to the problem of confirming that the activating cells were lymphocytes. Since the biological effects, particularly *in vivo*, of x-irradiation and sodium aurothiomalate are complex, the nature of these effects with regard to the immune system have been expounded in some detail below.

### 4.1 Effect of x-irradiation* on macrophage function

Certain macrophage functions such as phagocytosis and migration (Muramatsu, 1966) are generally regarded as being resistant to ionising radiation. Histological studies of irradiated macrophages failed to find evidence of damage (Brecher, Endicott, Gump and Brawner, 1948). Radiation does, however, induce several biochemical changes. Geiger and Gallily (1974) reported that DNA and RNA synthesis were elevated and choline uptake was enhanced in irradiated macrophages. Lysosomal enzyme levels in such cells are also elevated (Geiger and Gallily, 1974; Meyer and Dannenberg, 1970; Schmidtke and Dixon, 1973).

Phagocytosis, as assessed by clearance from the blood of bacteria (Benacerraf, 1960; Geiger and Gallily, 1974) and *in vitro* uptake of sheep red blood cells (Perkins, Nettesheim, and Morita, 1966) and radioiodinated protein antigens (Schmidtke and Dixon, 1972), is particularly insensitive to x-irradiation. However, post-phagocytic retention and degradation of antigen do appear to be affected in some

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*N.B. X-irradiation dose can be expressed in Roentgens (R) or rads (r). There is a slight difference in value between the two systems. Irradiation doses have been quoted as presented in the original papers in R or r.

studies (Donaldson, Marcus, Gyi and Perkins, 1956; Kakurin, 1959). In contrast, Perkins *et al.* (1966) found that digestion of sheep red blood cells by macrophages was unimpaired by irradiation and Schmidtke and Dixon (1973) found that *in vivo* irradiation 1 to 3 days before harvest of peritoneal macrophages did not affect the *in vitro* performance of these cells in handling keyhole limpet haemocyanin.

The capacity of macrophages to present antigen in a manner suitable for the induction of antibody formation is radioresistant for antigens such as sheep erythrocytes (Roseman, 1969; Goldie and Osoba, 1970; Gershon and Feldman, 1968) and with protein antigens the immunogenic capability of macrophages can even be heightened following irradiation (Schmidtke and Dixon, 1972). However, in studies with *Shigella* infection in mice the immunosuppression observed following sub-lethal (550r) x-irradiation was shown to be due in part to the inability of macrophages to process antigen (Gallily and Feldman, 1967). Similar studies by Pribnow and Silverman (1967) in rabbits, and by Mitchison (1969) in mice also showed impairment in the capacity of irradiated antigen-containing macrophages to transfer an immune response *in vivo*.

In studies using lymphoma cells, Den Otter, Evans and Alexander (1974) found that *in vitro* macrophage cytotoxicity was either radioresistant (not affected by doses in excess of 500R) or radiosensitive (activity abolished by 500R) depending on the manner in which the macrophage donor mice had been immunised. Radioresistant cytotoxic macrophages were obtained by immunising mice with living allogeneic lymphoma cells or, in a syngeneic system, by first immunising mice with radiation-killed tumour cells followed by challenge with live

tumour cells which are then rejected. Radiosensitive macrophages were found in both syngeneic and allogeneic mice immunised with radiation-killed lymphoma cells. In addition, the cytotoxicity of macrophages non-specifically activated *in vitro* by endotoxin or lipid A was resistant to doses up to 5,000R.

## 4.2 Irradiation and antibody production

The effects of *in vivo* x-irradiation on antibody production depend on a) the antigen, b) dose of irradiation and c) time of irradiation with respect to the antigenic challenge or infection. In general, immune responses are depressed when irradiation is given at or before antigenic challenge (Anderson and Warner, 1976). However, following antigen administration irradiation can lead to enhanced antibody production (Dixon and McConahey, 1963; Taliafero, Taliafero and Jaroslow, 1964). Taliafero *et al.*, (1964) proposed that this enhancement be classified as two types: type A, found with low doses (25-200r) and associated with heightened peak titre and shortened latent period; and type B, requiring exposures usually greater than 400r and associated with increased peak titre but lengthened latent period. Various mechanisms may be operating, not necessarily mutually exclusive, which may account for such enhancement. For example, lymphoid organs may be disproportionately repopulated with antigen-stimulated cells (Dixon and McConahey, 1963). The T cell suppressor system is particularly sensitive (*vide infra*)and this could lead to enhancement (Tada, Taniguchi and Okumura, 1971). A further mechanism which may contribute to this phenomena may lie in the observation of Sljivic (1970a, b) that following irradiation there

can be a transient bacteraemia by gut microbes leading to endotoxin stimulation of the reticuloendothelial system. This effect was not apparent in any of the thesis experiments using whole-body irradiation.

### 4.3 Irradiation and T cell helper and T cell suppressor function

There has been controversy in recent years over the sensitivity of T cell helper activity to x-irradiation. It is now clear, however, that the discordant results of different groups were due to fundamental differences in their experimental assays. *In vitro* assays using primed T cells indicate radioresistance whether irradiation is administered *in vitro* (Kettman and Dutton, 1971), or *in vivo* (Katz and Unanue, 1973). Adoptive transfer assays with primed T cells, on the other hand, generally indicate radiosensitivity of helper functions (Anderson *et al*., 1974; Hamaoka *et al*., 1972; Janeway, 1975). The discrepancy between the two assay systems is explained by changes in the *in vivo* migratory capacity of irradiated lymphocytes which fail to home to lymphoid organs (Anderson *et al*., 1974; Hamaoka *et al*., 1972). Studies utilising an *in situ* irradiation procedure in which homing is not required also show radioresistance of helper activity. Non-antigen-specific T cell-mediated help is also radioresistant (Hirst and Dutton, 1970; Gershon, Leibhaber and Ryu, 1974). Unprimed T cells have invariably been found to be radiosensitive (Miller and Mitchell, 1968; Ito and Cudkowicz, 1971).

Suppressor T cells appear to be radiosensitive (Basten *et al*.,

1974; McCullagh, 1975). However, some experimental models suggest that once activated, suppressor cells are more resistant. For example, the *in vitro* suppression of antibody formation to sheep erythrocytes by concanavalin A-activated T cells was resistant to 2,000R (Dutton, 1972; Rich and Pearce, 1973) but irradiation of the T cells before exposure to mitogen abrogated the appearance of suppressor effects. This would suggest that there are suppressor T cell sub-populations consisting of radiosensitive precursors and radioresistant effectors. However, suppressor activity is not sensitive to mitomycin C treatment (McCullagh, 1974; Basten *et al*., 1975) indicating that cell proliferation does not follow suppressor cell activation. An alternative explanation of radioresistance of suppression is that it is mediated by macrophages passively armed by activated T cells (Asherson and Zembala, 1974; Bash and Waksman, 1975).

### 4.4 Irradiation and cytotoxic lymphocytes

As with cytotoxic macrophages (*vide supra*) cytotoxic lymphocytes comprise two categories: one sensitive to irradiation doses of about 500r, and the other resistant to doses up to 2000r (Denham *et al*., 1970). Antigen-stimulated lymphocytes appear to be more radioresistant (Grant *et al*., 1972; Denham *et al*., 1970).

### 4.5 X-irradiation and in vitro macrophage activation

In the light of the foregoing brief review on the effects of ionising radiation on the immune response, the following interpre-

tations have been made on the results of experiments using x-irradiation to analyse the *in vitro* activation of macrophages.

The *in vitro* irradiation of spleen cells prior to their addition to adherent PE cells showed that the activating cell population(s) was sensitive to even the lowest dose used, 200r. Small lymphocytes in general are radiosensitive and *in vitro* doses of as little as 2-5R can result in changes in motility and morphology (Stefani and Schrek, 1964). Most, if not all, irradiated cells are subject to mitotic death at the first or second post-irradiation mitosis but some such as non-activated small lymphocytes, including B cells, are subject to interphase death. These effects can be manifest very soon (within one hour) following irradiation (Cronkite, Chanana, Joel and Laissue, 1974; Bari and Sorenson, 1964; Jordan, 1967). Thus the macrophage activating cells could comprise a population of non-activated precursor cells which may require cell division to mediate their effects. B cells or monocyte precursors could be involved.

This radiosensitivity of the activating cell population also confirmed that the observed anti-tumour effects were not due to cytotoxic spleen cells persisting in culture wells since Woodruff *et al.* (1976b) demonstrated that the *in vitro* anti-tumour cytotoxicity of spleen cells from *C. parvum*-treated mice was resistant to 800r.

*In vitro* irradiation of PE cells prior to seeding in culture plates had no effect at the lower doses, 200r and 400r, although there was a slight depression of activation at 800r. This supported the view that the mature macrophage population of adherent PE cells was the source of anti-tumour effector cells in the assay. Other

radioresistant cells such as activated cytotoxic T cells or helper T cells may also have been present in the wells but it is difficult to see how they could significantly aid the activation of macrophages by lymphoid cells from C. parvum-treated animals.

In the experiments investigating the maintenance of cytotoxicity of C. parvum-stimulated adherent PE cells, in vitro irradiation of PEC also had no effect (Table 22). However, in this situation the presence of a population of C. parvum-primed helper cells (and, therefore, radioresistant) could be involved in the reactivation of macrophages.

The experiments using in vivo irradiation proved more difficult to analyse. Whole-body x-irradiation doses of 200r and 400r given just before i.p. C. parvum, reduced the capacity of spleen cells to activate normal macrophages in vitro 8 days later. A third group of mice given 800r died or were too sick to use. It would be interesting to know if activation could be completely abrogated by other irradiation protocols such as using higher doses or irradiating at different times with respect to C. parvum administration. A complication of the above experiment was that the 8 day delay following irradiation would allow some recovery of the mouse's response to C. parvum.

Other workers have also studied the effects of x-irradiation on the anti-tumour activity of C. parvum. Woodruff et al., (1976b) using similar experimental conditions and materials to those used here, found that whole-body x-irradiation doses of 400r given either just before, or 4 days before C. parvum, abolished the in vitro cytotoxicity of PE cells. Similarly with spleen cells, cytotoxicity

could be ablated by 400r when given 4 days before C. parvum, but 800r was required for this effect when given just before C. parvum injection. In addition, it was observed that the cytotoxicity of spleen and PE cells was resistant to whole-body irradiation given after activation of cytotoxic cells had occurred.

In the same study, (Woodruff et al., 1976b) it was also shown that the effects of systemic C. parvum on a growing tumour nodule could be greatly reduced by irradiating the mice either 4 days before or 4 days after C. parvum injection. Even the strong anti-tumour effect which follows injection of C. parvum at the site of a tumour was ablated by 500r irradiation 4 days prior to C. parvum. Similarly, Milas et al. (1974d) showed that irradiation prior to, but not following, treatment with Corynebacterium granulosum abrogated this organism's in vivo anti-tumour effects.

These results thus confirm that the anti-tumour activity of C. parvum is dependent on a population of radiosensitive cells, a population which could include macrophage precursors, and unprimed T cells and B cells. Radiation effects on the function of non-dividing cells, such as the degradation and presentation of C. parvum antigens, could also play a part.

### 4.6 Effect of gold salt on macrophage activation

Gold compounds have been shown to have several effects on macrophages. Sodium aurothiomalate is rapidly taken up by phagocytic cells resulting in the inhibition of lysosomal enzyme activity in these cells (Persellin and Ziff, 1966), apparently by reversibly binding to sulphydryl groups (Ennis et al., 1968). Phagocytosis

and migration of macrophages are also suppressed by this compound (Jessop et al., 1973; Vernon-Roberts et al., 1973).

Sodium aurothiomalate has been shown to abrogate the protective effect of C. parvum treatment in mice to tumour challenge (McBride et al., 1975b; James et al., 1976). Multiple gold salt injections also reduced the in vitro anti-tumour capacity of peritoneal exudate cells from mice treated with C. parvum 4 days previously and to a lesser extent 7 days previously (Ghaffar et al., 1976b). The recovery of cytotoxicity in the peritoneal cell population between day 4 and day 7 may have been due to the continuing stimulation of the reticuloendothelial system by C. parvum, known to persist in the lymphoid organs of the mouse for periods up to a month following injection (Sadler, Cramp and Castro, 1977; Scott and Milas, 1977; Dimitrov, Greenberg and Denny, 1977).

That this interference with the anti-tumour activity of C. parvum by sodium aurothiomalate was directed atthe effector level and not at the process leading to macrophage stimulation was illustrated by the fact that anti-tumour cytotoxicity of in vivo C. parvum-stimulated macrophages could be blocked by the presence of gold salt in vitro (Ghaffar et al., 1976b). This effect was also noted in this thesis where the presence of gold salt either during macrophage adherence or during activation abrogated the anti-tumour effects (Table 9). It is also relevant that the continuous stimulation of cytotoxic macrophages by the addition of C. parvum in vitro was sensitive to the presence of gold salt (Part 2, Table 23).

This result and those of others discussed above suggest that macrophage lysosomal enzymes contribute to the anti-tumour effects

of *C. parvum*. Furthermore, the observation that *in vivo* administration of sodium aurothiomalate prevented the appearance of spleen cells capable of activating macrophages *in vitro* indicates that intact macrophage function is necessary in this process, presumably via the phagocytosis of *C. parvum* and presentation of its antigens to lymphocytes. Several other immunological consequences of treatment have been reported that may involve the macrophage. For example, it prolongs survival time of skin homografts in mice (Scheiffarth, Baenkler and Islinger, 1970) and inhibits the PHA response of lymphocytes (Cahill, 1971). Multiple injections of sodium aurothiomalate also prevent the appearance of serum immunoglobulins capable of binding to tumour cells in both tumour-bearing and *C. parvum*-treated tumour-bearing mice (James *et al*., 1976). This study also recorded that gold treatment prevented the elevation of serum levels of IgM, IgG2a and IgG2b that follow systemic *C. parvum* administration. Similarly, McBride *et al*. (1975b) reported that multiple injections of sodium aurothiomalate partially inhibited the immune response to sheep red blood cells and to *C. parvum* itself in *C.* parvum-treated mice. Gold has been shown to bind to serum proteins, principally albumin and, at higher serum concentrations, also to immunoglobulin and complement, a phenomenon which may aid the uptake of gold by macrophages (Lorber, Bovy and Chang, 1972). As discussed below immunoglobulin, complement and antigen-antibody complexes may play a significant role in *C. parvum*'s anti-tumour activity as mediated by macrophages.

In contrast to the above results several immune mechanisms, which are probably macrophage independent, are either unaffected by

or stimulated by gold treatment. For example, although there is an increased plaque-forming cell response to sheep red blood cells (Scheiffarth, Baenkler and Pfister, 1971; Measel, 1975), there is no effect on antibody response to Semliki Forest virus (Allner, Bradish, Fitzgeorge and Nathanson, 1974),and no effect on the delayed hypersensitivity response of guinea pigs (Persellin, Smiley and Ziff, 1967).

### 4.7 Anti-macrophage serum (AMS)

The finding that anti-macrophage serum (AMS) treatment of spleen cells failed to affect their capacity to activate normal macrophages *in vitro*, provided further confirmation that the activating cells were not macrophages (table 7a). On the other hand, the same batch of AMS was able to abrogate the direct *in vitro* anti-tumour cytotoxicity of unseparated spleen cells from *C. parvum*-treated mice (table 7b). This demonstrated that, under the conditions of the former experiment (table 7a), splenic macrophages carried over into the culture wells and persisting there, could not be contributing significantly to the observed cytotoxic effects following the activation process.

## 5. FURTHER ANALYSIS OF THE MACROPHAGE-ACTIVATING LYMPHOCYTES

The lymphocytes involved in *in vitro* macrophage activation were further characterised in a series of experiments which provided evidence for the participation of both T cells and B cells.

## 5.1 T cell evidence

The capacity of spleen cells from thymically-intact mice and from T cell-deprived mice to activate macrophages was investigated at various times following i.p. injection of *C. parvum*. In agreement with the work of Bomford and Christie (1975), the results indicated that T cell deprivation severely restricted the development of spleen cells capable of activation, although the slight anti-tumour activity at day 12 suggested that such development may only be delayed in T cell-deprived mice. It is possible that greater cytotoxicity would have ensued if spleen cells had been harvested at some time later than day 12.

Thymectomised, irradiated, bone marrow reconstituted mice are probably not totally devoid of T lymphocytes and so the result at day 12 may reflect expansion of a residual host T cell population in the donor bone marrow under the stimulus of the *C. parvum* injection. Alternatively, there may be what Howard, Scott and Christie (1973) have termed a 'T cell bypass' mechanism operating in these mice. The stimulation of B cells in T cell-deprived mice, together with retention of some T cell helper activity, may be sufficient for the macrophage activation process to occur. It would be interesting to repeat this experiment using an extended time course in both 'B' mice and nude mice.

In contrast, there have been many reports demonstrating the apparent non-T cell dependency of the anti-tumour action of *C. parvum in vivo* (Woodruff *et al*., 1973; Sadler and Castro, 1976; Woodruff and Warner, 1977). *In vitro* assessment of spleen and peritoneal

exudate cell cytotoxicity has also shown that *C. parvum* is as effective in T cell deprived mice and homozygous nude mice as in thymically intact mice. The problem of residual T cell activity also plagues analysis of these experiments.

Another observation which may be important in an analysis of mechanisms is that although cells cytotoxic for tumour cells *in vitro* appear in peritoneal exudate by day 4 in thymically intact mice, 'B' mice and homozygous nude mice (Ghaffar *et al*., 1975), the cytotoxic capacity of spleen cells from T cell-deprived mice is more fully developed at day 3 than that of intact mice (table 13). This earlier appearance of cytotoxic spleen cells in the 'B' mouse may have been due to a lack of suppressor cells in these mice. A glass-adherent splenic population of cells which suppressed responsiveness to PHA, concanavalin A and the mixed lymphocyte reaction, has been isolated from rats by Folch and Waksman (1974a, b). The suppressor activity was absent in spleens from T cell-deprived rats and it was concluded that the suppressor cells were T cells or were dependent on the presence of T cells for their activity. Splenic suppressor cells, believed to be macrophages, and which inhibited proliferation of T cells in the presence of PHA, have been detected in *C. parvum*-treated mice (Scott, 1972a, b; Kirchner, Holden and Herberman, 1975). Suppression in tumour-bearing hosts has been attributed to T cells (Fujimoto, Greene and Sehon, 1976a, b) and to macrophages (Kirchner, Glaser, Holden *et al*., 1976). Gorczynski (1974) has attributed suppressor activity to B cells. It has been suggested that suppression may in fact be mediated by macrophages in response to a T cell signal (Taylor and Basten, 1976). Whatever the mechanism,

suppressor systems must play an important part in the modulation of the host's response to C. parvum or a growing tumour.

Experiments in which spleen cells were treated with anti-θ serum and guinea pig complement provided no conclusive support for the involvement of T cells in macrophage activation. Interpretation of results was made difficult by the abrogating effect of the normal serum controls, despite their known low anti-θ activity (table 10). This effect was also found in a recent experiment (data not shown) using, as a control, anti-θ serum which had been neutralised by absorption with $C_3H$ mouse brain. The reduction in activation by serum treatment (control and anti-θ) may have been due to non-specific blocking of B cell Fc receptors. Antigen-antibody complexes, for example, can inhibit the antibody response both in vitro and in adoptive transfer experiments (Sinclair, Lees, Abrahams et al., 1974; Hoffmann, Kappler, Hirst and Oettgen, 1974). Antibody-dependent cell mediated cytotoxicity can be inhibited by antigen-antibody complexes (MacLennan, 1972b), normal serum (MacLennan, Loewi and Harding, 1970), Ig (Scornik, Cosenza, Lee, Kohler and Rowley, 1974; Greenberg, Shen, and Roitt, 1973), and heat-aggregated IgG (MacLennan, 1972b). The sera used in this study had not been ultracentrifuged or refined in any way, and so aggregates and complexes may have been present.

It should be noted that Christie and Bomford (1975) found that anti-θ serum treatment of spleen cells from C. parvum-treated mice did abrogate their capacity to activate macrophages in vitro. Several other workers have found that in vitro macrophage activation was T cell dependent (Evans et al., 1972; Lohmann-Matthes and

Fischer, 1973; Lohmann-Matthes et al.,1973).

5.2 B cell evidence

The observation that normal AKR serum, together with guinea pig serum, could block the macrophage activating capacity of spleen cells suggested that B cells may also be involved in activation. Further evidence supporting this idea derived from experiments in which spleen cells were separated on nylon wool columns into B cell enriched and B cell depleted fractions. Depletion of B cells (more precisely, Ig-bearing cells) in spleen cell suspensions greatly reduced the level of cytotoxicity in the subsequent assay. Use of the nylon adherent, B cell enriched population in the assay resulted in almost the same degree of activation as obtained with unfractionated spleen cells.

No functional T cell and B cell tests were carried out on the separated fractions and it was assumed from the work of others (Julius et al., 1973; Handwerger and Schwartz, 1974; Trizio and Cudkowicz, 1974), that such functions would be unaffected by incubation in nylon wool. However, as a recent report has shown, some changes can occur, at least in nylon wool treated thymocytes, such as altered surface morphology and depressed cyclic adenosine monophosphate levels (Kwock et al., 1976).

This involvement of B cells in macrophage activation has been demonstrated by other researchers (Wilton, Rosenstreich and Oppenheim, 1975). A macrophage-B cell cooperation has also been described, in a syngeneic tumour system, in which macrophages lysed tumour cells in the presence of an anti-tumour antibody of the IgG2a class (Yamazaki,

Shinoda and Mizuno, 1975, 1976; Yamazaki, Shinoda, Suzuki and Mizuno, 1976). Antibody-dependent macrophage-mediated cytotoxicity presents an interesting possibility as an alternative explanation for the observations in this thesis. In the experiments using supernatants from spleen cell cultures, antibody to C. parvum may have been produced and could therefore mediate a cytotoxic effect by binding to macrophages or, as suggested by the recent work of James et al. (1976, 1977) to tumour cells. Direct binding of immunoglobulins to tumour cells would be less likely since supernatants are removed and the culture wells washed before tumour cells are added. However, without being able to formally exclude the possibility, it seems unlikely that antibody-dependent macrophage-mediated cytotoxicity can account for the observed results in view of the inability of sera from C. parvum-treated mice to confer any cytotoxic potential on normal macrophages.

## 6. MACROPHAGE ACTIVATION BY FACTORS RELEASED BY LYMPHOCYTES

Experiments using supernatants from cultures of spleen cells established that macrophage activation could be effected by a factor, or factors, released by C. parvum-immune spleen cells in response to the presence of C. parvum in the cultures. Such factors are generally referred to in the literature as macrophage activating factors (MAF) although some authors have used the abbreviations MCF (macrophage cytotoxicity factor) (Lohmann-Matthes et al., 1972) or SMAF (specific macrophage arming factor) (Evans and Alexander, 1972a, b). Treatment of macrophages with SMAF resulted in a state of preactivation called "arming" and only subsequent exposure of such macrophages to the

sensitising antigen resulted in activation (Evans and Alexander, 1972a, b).

Normal (non-cytotoxic) macrophages have been rendered cytotoxic to tumour cells by incubation with MAF derived from cultures of syngeneic (Evans and Alexander, 1971; Fidler, 1975; Churchill, Piessens, Sulis and David, 1975), allogeneic (Evans and Grant, 1972; Lohmann-Matthes, Ziegler and Fischer, 1973; Pels and Den Otter, 1974), or xenogeneic (Fidler, 1975; Fidler, Darnell and Budmen, 1976) lymphocytes. MAF has also been described in an allograft model (Dimitriu, Dy, Thomson and Hamburger, 1975) and in studies on the anti-microbial role of macrophages (Patterson and Youmans, 1970; Krahenbuhl and Remington, 1971; Godal, Rees and Lamvik, 1971). MAF-rich supernatants were generally obtained in these studies from cultures of sensitised lymphocytes incubated with the sensitising antigen, and treatment of macrophages with the supernatants resulted in stimulated macrophages which were specifically, or non-specifically cytotoxic depending on the system studied. It would appear that previous exposure of animals to pathogens results in a state of preactivation such that only non-specific MAF is produced (Evans and Alexander, 1972a; Lohmann-Matthes, 1976) and severe infection can lead to the production of macrophages which are cytotoxic without the need for further _in vitro_ activation (Hibbs _et al_., 1971, 1972; Krahenbuhl and Remington, 1974; Keller and Jones, 1971; Evans and Alexander, 1972a). Thus specific pathogen free animals ought to be used to ensure production of specific macrophage activating factor. Non-specific MAF has also been obtained from mixed lymphocyte cultures (Godal _et al_., 1971; Dimitriu _et al_., 1975) and from concanavalin

A-stimulated lymphocyte cultures (Fidler, Darnell and Budmen, 1976).

Fractionation of MAF-containing supernatants by column gel-filtration has been performed by several groups, and the molecular weight determinations all fall within the same range: 35,000-55,000 Daltons (Nathan _et al_., 1973), about 30,000 Daltons (Lohmann-Matthes, 1976) and in an allograft situation, 45,000-55,000 Daltons (Dy, Kamoun, Dimitriu and Hamburger, 1976). Evans _et al_. (1972) reported that in addition to a low molecular weight fraction (50,000-60,000) MAF activity was also associated with a fraction containing material of a size greater than 300,000 Daltons. The lower molecular weight material elutes in the same band as other non-antibody lymphocyte mediators called lymphokines (Dumonde _et al_., 1969, 1975). Nathan, Remold and David (1973) found that macrophage migration inhibitory factor eluted from Sephadex G-100 in the same fraction as MAF, and in fact these authors were unable to distinguish between MIF and MAF, concluding that they may be the same material. MIF/MAF activity could be differentiated from other lymphokines eluting in this fraction such as lymphotoxin and macrophage chemotactic factor (Nathan _et al_., 1973). This association between MIF and MAF has also been observed by others (Rocklin, Winston and David, 1974b; Lohmann-Matthes, 1976) although further analysis is required to determine whether they are indeed the same substance.

Another lymphokine which is potentially capable of mediating the cytotoxic effects in the anti-tumour assay is interferon, known to activate macrophages (Schultz, Papamatheakis and Chirigos, 1977a) and to inhibit cellular proliferation (Gresser, Brouty-Boye, Thomas

et al., 1970). Interferon is produced in vitro by non-adherent spleen cells, possibly B cells, from C. parvum-treated mice and, provided that C. parvum is present, also from untreated mice (Hirt, Kochen and Kirchner, 1977). This production of interferon by normal spleen cells makes this lymphokine a less likely candidate for the mediator molecule in the system described in this thesis since normal spleen cells did not activate macrophages.

There have been several attempts to identify the lymphocyte responsible for the production or release of MAF. Studies using anti-θ serum (Evans et al., 1972; Grant et al., 1973; Lohmann-Matthes et al., 1973), T cell-deprived mice (Evans et al., 1972), and cell separation on cotton wool columns (Lohmann-Matthes and Fischer, 1973) indicated that the macrophage activating factor was T cell-dependent and may have been a T cell product. However, other workers have attributed MAF production, as assessed by the enhanced incorporation of $^{14}$C-labelled glucosamine into macrophages in culture (Hammond and Dvorak, 1972; Wilton, Rosenstreich and Oppenheim, 1975), to both T and B cells, following stimulation with antigens or mitogens (Wahl, Wilton, Rosenstreich and Oppenheim, 1975). Wahl and Rosenstreich (1976), however, believe that antigen-specific lymphokine synthesis by B cells requires help by a soluble factor from antigen-activated T cells. Wahl et al. (1975) also observed that whereas T cell production of monocyte chemotactic factor and MAF required the cooperation of macrophages, B cell production of these lymphokines could occur in the absence of macrophages. The possible role of macrophages in the release of mediators by lymphocytes has yet to be assessed in the in vitro C. parvum anti-tumour assay.

Other mediators once thought to be solely of T cell origin have now also been demonstrated to be elaborated by B cells: macrophage migration inhibitory factor (MIF) (Papageorgiou, Henley and Glade, 1972; Rocklin, MacDermott, Chess et al., 1974a); osteoclast activating factor (Mundy, Luben, Raisz et al., 1974); interferon (Epstein, Kreth and Herzenberg, 1974; Wallen, Dean and Lucas, 1973); and lymphotoxin (Granger et al., 1970). Of particular interest is the finding that antigen-antibody complexes and bacterial products such as LPS and tuberculin PPD could stimulate B cell enriched populations to produce a macrophage chemotactic factor (Wahl, Iverson and Oppenheim, 1974). It is thus conceivable that C. parvum, known to be a B cell mitogen (Zola, 1975) would also stimulate B cell production of macrophage-regulating lymphokines.

The nature of the specific component of specific MAF has been the subject of much speculation. The discovery of Evans et al., (1972) that SMAF activity was also associated with a large molecular weight ( 300,000 Daltons) substance suggested that specificity could be due to an immunoglobulin. Indeed SMAF can be absorbed from culture supernatants by macrophages or the immunising tumour cells in an antibody-like manner (Evans et al., 1972; Pels and Den Otter, 1974). However, Lohmann-Matthes and Fischer (1973) demonstrated that the specificity of their activating factor was not due to conventional IgG or IgM, to antigen/antibody complexes, or to cooperation between a non-specific T cell product and antibody. Furthermore, a recent analysis of a non-specific MAF, which also gave two active fractions, one of 60,000 Daltons and one greater than 300,000 Daltons, failed to detect IgM in the larger molecular

weight material (McDaniel, Laudico and Papermaster, 1976). MAF activity in the two fractions was associated with albumin and alpha-2-macroglobulin (alpha-2-M) respectively, and it was mooted that these proteins were acting as carriers for the activating factor. It should be noted that this particular MAF was obtained from a human cultured lymphoid cell line and thus may not be typical of MAF produced by lymphocytes responding to antigen or mitogen. Nevertheless, the idea that alpha-2-M may be associated with macrophage activation is very interesting in light of the diverse range of homeostatic and immunological functions already attributed to this serum protein (James, 1975; Horne, Thomson, Towler et al., 1975; Debanne, Bell and Dolovich, 1976; Blumenstock, Saba, Weber and Cho, 1976; Harpel, 1973).

Owing to the lack of positive evidence for a role for B cell antibody in specific MAF preparations, and because of its T cell-dependency, the possibility was considered that T cell immunoglobulin could be involved. Evidence has accumulated showing that T cells carry, on their surface, immunoglobulin determinants (Bankhurst, Warner and Sprent, 1971; Marchalonis, Haustein, Harris and Miller, 1975) known to resemble the monomeric subunits of IgM pentamers (Marchalonis, Cone and Atwell, 1972), and which can act as antigen receptors (Marchalonis and Cone, 1973). In this respect it is intriguing that the operation of specific MAF can resemble the stimulation of B cell responses to antigen by T cells, a cooperation which is mediated by macrophages which have absorbed complexes of T cell-Ig and antigen released from the surface of the activated T cells (Feldmann, 1972; Cone, Feldmann, Marchalonis and Nossal, 1974). It is also relevant that the T cell-Ig in this work was cytophilic for macrophage whereas

B cell monomeric IgM was not (Cone et al., 1974). MAF is cytophilic for macrophages (Lohmann-Matthes and Fischer, 1973; Evans and Alexander, 1976).

The question of whether C. parvum-elicited MAF is of the specific or non-specific variety is difficult to determine, at least from the evidence presented in this thesis. Since the mice used were from an open colony it might be expected that the MAF produced would be non-specific (Lohmann-Matthes, 1976). Indeed non-specific activation appears to occur in that once macrophages have been incubated in MAF-containing supernatant, C. parvum need not be added to achieve anti-tumour cytotoxicity. A similar result was reported by Evans et al., 1972 using BCG, but unfortunately, in both studies soluble BCG or C. parvum antigens may have been present in the supernatants used. On the other hand Christie and Bomford (1975), although obtaining some activation using C. parvum-elicited MAF alone, found that the greatest anti-tumour effect resulted when macrophages were exposed to MAF in the presence of C. parvum, or even unrelated bacteria such as Proteus vulgaris and Escherichia coli. These authors thus demonstrated that the production of C. parvum-MAF was a specific event but that once exposed to MAF macrophages could be activated to full cytotoxicity by other (non-specific) bacteria.

The question of the specificity of C. parvum-MAF must remain an open one particularly in view of the evidence (discussed more fully in an earlier section on specificity) that both specific and non-specific anti-tumour responses occur following the systemic administration of C. parvum.

Although MAF production can be demonstrated easily in vitro it

doesn't necessarily follow that it is an important effector mechanism in vivo. The inability of sera from C. parvum-treated mice to activate macrophages in vitro suggests either that it did not contain MAF or that it was present in insufficient quantities. Thus in vivo MAF production may only be a local phenomenon, possibly confined to lymphoid organs, and requiring close association of lymphocytes, macrophages and C. parvum. In this respect it would be important to know the distribution of C. parvum following its injection. Three recent studies (Dimitrov et al., 1977; Sadler et al., 1977; Scott and Milas, 1977) using fluorescein-labelled and/or radio-labelled C. parvum showed that following i.p. or i.v. injection C. parvum could be located in the liver, spleen, gut and, to a lesser extent, in bone marrow, lungs and lymph nodes. Subcutaneous administration of C. parvum resulted in a persistence of the bacteria at the site of inoculation, thus probably accounting for the poor efficacy of this route of administration in treating any but local tumour nodules.

These studies were also able to throw further light on the mechanism of C. parvum's anti-tumour effects, and are worth elaborating in more detail. Scott and Milas (1977) found that in contrast to a biologically inactive strain of C. parvum (CN 5888) which was rapidly broken down and cleared, C. parvum 6134 persisted in organs, and could be detected after more than a month following injection. This resistance to degradation of C. parvum 6134 is probably important in its anti-tumour effects, and would also account for its hyperplastic effect on liver and spleen (Adlam and Scott, 1973).

Labelled C. parvum could also be located in the body of solid tumours (Sadler et al., 1977; Scott and Milas, 1977) although there

was no correlation between the amounts found and the susceptibility of tumours to i.v. administered C. parvum. Autoradiographic studies confirmed that labelled C. parvum was only present in the cytoplasm of phagocytic cells in the various organs examined (Sadler et al., 1977).

These results, therefore, show that macrophage activation could occur locally and over an extended period of time. It is also interesting that although no C. parvum was found in peritoneal exudate cells after i.v. injection, the peritoneal macrophages had, nevertheless, been activated and could inhibit tumour growth in vitro (Scott and Milas, 1977). Presumably macrophages, or their precursors, were being activated elsewhere before migrating into the peritoneal cavity. Furthermore, the absence of labelled C. parvum in these cells meant that, either phagocytosis of the bacteria was not required in this process, or that they were digested very rapidly following activation. It has been observed that bone marrow-derived macrophages can be activated in vitro by MAF (Meerpohl, Lohmann-Matthes and Fischer, 1976).

In conclusion, there are various areas for further research with respect to C. parvum-MAF. It is not known whether the producing lymphocytes are B cells, or T cells or both and, although the spleen cells in this study were depleted of glass-adherent cells, this procedure is not rigorous enough to exclude the possible involvement of macrophages in MAF release. The specificity of C. parvum-MAF could be explored further by replacing C. parvum 6134 with other strains and other organisms. MAF-rich supernatants should also be fractionated on Sephadex columns to determine the molecular weight of the active moiety.

## 7. DIRECT ACTIVATION OF MACROPHAGES BY C. PARVUM

The experiments attempting to activate normal, non-cytotoxic peritoneal macrophages by culturing them with *C. parvum*, serum from *C. parvum*-treated mice or with mixtures of both to give antigen-antibody complexes, all proved unsuccessful. This approach had been prompted by reports of direct activation *in vitro* of macrophages by various agents; a fraction extracted from *Mycobacterium smegmatis* (Juy and Chedid, 1975), double-stranded RNA, the lipid A fraction of endotoxin, synthetic poly(I).poly(C) (Alexander and Evans, 1971; Parr, Wheeler and Alexander, 1973), and possibly tumour antigen-antibody complexes (Evans, 1975). Nevertheless, the failure to demonstrate a direct activation by *C. parvum* *in vitro* does not rule out the possibility of such a mechanism operating *in vivo*, possibly mediated by humoral factors such as complement and antibodies to *C. parvum*.

*C. parvum* has been shown to activate both the classical and alternate (properdin) pathways of complement when added to guinea pig serum (McBride, Weir, Kay *et al*., 1975c). Activation of both pathways can also occur in patients receiving *C. parvum* immunotherapy (Biran, Moake, Reed *et al*., 1976). Activation of the alternate pathway (Gotze and Muller-Eberhard, 1971) has also been described for endotoxic lipopolysaccharides exhibiting anti-tumour activity (Okuda, Yoshioka, Ikekawa *et al*., 1972).

The importance of the lipid or lipopolysaccharide content of the anaerobic coryneform bacteria is now also recognised as it apparently accounts for many of the biological properties of these organisms: increased blood clearance of carbon (Otu, Russell and White, 1977) and virulent organisms (Fauve and Hevin, 1974); increased resistance towards

infection (Fauve and Hevin, 1974); anti-tumour activity (McBride, Dawes and Tuach, 1976); chemoattractant for macrophages (Russell, McInroy, Wilkinson and White, 1976). It has been proposed that the biological activities of endotoxic lipopolysaccharides are mediated by the complement system (Gewurz et al., 1968). Such a system might explain why such a great diversity of agents and microorganisms can have similar effects on macrophages.

Thus a mechanism of macrophage stimulation can be envisaged during C. parvum treatment requiring activation of the alternate pathways or, following formation of C. parvum-C. parvum antibody complexes, of the classical pathway and probably involving binding of complexes at macrophage C3 receptors. Also of great relevance to the activation of macrophages by C. parvum is the observation of Ogmundsdottir and Weir (1976) that C. parvum NCTC 10390 binds to mouse peritoneal macrophages via plasma membrane receptors having specificity for sugar determinants in the cell wall of C. parvum bacteria, whereas the biologically inactive anaerobic coyneform Propionibacterium freudenreichii NCTC 10470 exhibited only slight binding. Trypsin treatment of the macrophages failed to affect this binding indicating that the receptors were not cell-bound antibody.

The association of complement and the activation of anti-tumour activity in macrophages by C. parvum can be extended further. Attachment of the C3 cleavage product, C3b, to mouse macrophages in vitro results in a release of lysosomal enzymes. Such enzymes can also cleave C3 thus generating more C3b and creating an amplification system (Schorlemmer, Davies and Allison, 1976). In addition a fragmentation product of the fifth component of complement C5a, has

been shown to be an important chemotactic factor for guinea pig macrophages and polymorphonuclear leukocytes (Snyderman, Shin and Hausman, 1971). C5 can be cleaved by macrophage-derived proteinase to give several chemotactic fragments thereby providing amplification (Snyderman, Shin and Dannenberg, 1972).

Activation of both the alternate pathway and, by virtue of the presence of naturally occurring antibodies to *C. parvum* in the mice used in these studies (Woodruff, McBride and Dunbar, 1974; James *et al*., 1976), the classical pathway could occur soon after *C. parvum* treatment. Thus complement may play an essential role in the earlier effects of *C. parvum* on the reticuloendothelial system. Among such early consequences is a stimulation of macrophage colony-forming cells from bone marrow in normal mice (Wolmark *et al*., 1974; Baum and Breese, 1976) and in tumour-bearing mice (Wolmark and Fisher, 1974). The numbers of colony-forming cells reached a maximum 48 hours following *C. parvum* treatment. However, in a similar study, Dimitrov, André, Eliopoulos and Halpern (1975) found that the effect was not apparent until 5 days after inoculation. Macrophages derived from the bone marrow of tumour-bearing mice exhibit specific anti-tumour cytotoxicity *in vitro* (Fisher, Wolmark, Coyle and Saffer, 1976c). Bone marrow macrophage precursors from normal mice can also be rendered cytotoxic by a macrophage cytotoxicity factor derived from cultures of immune lymphocytes (Meerpohl, Lohmann-Matthes and Fischer, 1976). (*vide supra*).

Another early effect following systemic *C. parvum* treatment is the appearance, by day 3 or 4, in the spleen and peritoneal cavity, of macrophages cytotoxic for tumour cells *in vitro* (this thesis, Ghaffar

et al., 1974).

It is of interest that a recent publication describes a biphasic activation of murine macrophages, as assessed by carbon clearance, following i.p. C. parvum treatment (Otu et al., 1977). There was a peak of activity at 2 days, attributed to the lipid content of the bacteria, followed by a late phase of activation with maximal activity at 14 days, the reasons for which were less clear. This later peak of stimulation may be explained by the production of macrophages activated by lymphocytes responding to the C. parvum injection as described in this thesis.

## 8. MECHANISMS OF MACROPHAGE ACTIVATION

It is easy toforget in immunobiology that when studying an arm of the immune system in isolation, that that arm is, in reality, only part of a complex, but integrated, balanced whole. Administration of immunopotentiators such as C. parvum result in an alteration of the balance, and all aspects of the immune system are affected: the reticuloendothelial system is stimulated, there is a strong adjuvant effect on B cells, many T cell-mediated phenomena are depressed and both specific and non-specific anti-tumour responses augmented. This thesis has been primarily concerned with the anti-tumour effects of C. parvum as mediated by activated macrophages and in the mechanisms underlying the production of such macrophages.

A picture has emerged of two processes leading to the development of macrophages with in vitro anti-tumour potential, one an early phase, possibly involving direct activation of mature macrophages

or macrophage precursors, and the other, a later phase of activation, mediated by lymphocytes sensitised to C. parvum. The first mechanism possibly operates concurrently with the second once it becomes established. These proposed mechanisms have been outlined below and are shown diagrammatically in fig. 7. Direct activation could occur by binding and/or ingestion of C. parvum or of C. parvum-antibody complexes. No *in vitro* evidence was obtained for such a concept. However, *in vivo* activation of the complement system by the classical or alternate pathways could be associated with such a mechanism. Complement activation would also result in the production of complement derived macrophage chemotactic and macrophage stimulatory factors as well as cleavage products of C3 known to be cytolytic for tumour and other cells.

A known early effect of C. parvum is the stimulation of bone marrow colony formation. Whether this effect is mediated by C. parvum directly or by humoral factors such as antibody and complement is not known. It would be interesting to see if bone marrow cells could be stimulated to form colonies *in vitro* by C. parvum alone or whether serum from C. parvum-treated mice was required. The *in vitro* cytotoxicity of such colony cells could also be assessed. Bone marrow-derived macrophage precursors have been activated *in vitro* by a MAF preparation (Meerpohl *et al*., 1976).

Early activation of macrophages may be mediated by the lipid component of C. parvum, as suggested by Otu *et al*., (1977).

Spleen cell-mediated macrophage activation did not appear until day 6 or 7 post i.v. or i.p. injection of C. parvum which implied that an immunological process was involved. This was also shown

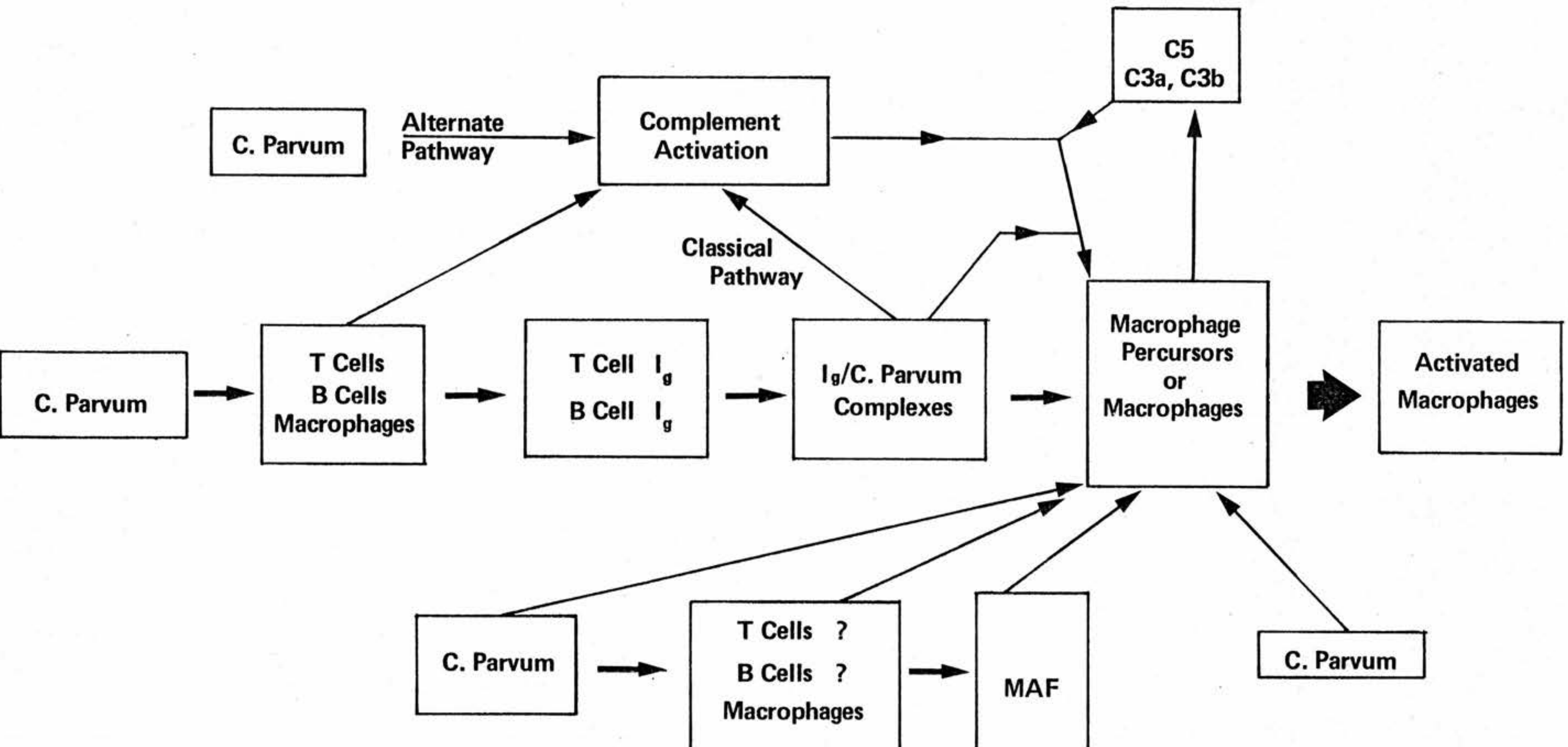


**Figure 7.** **MECHANISMS OF MACROPHAGE ACTIVATION FOLLOWING SYSTEMIC ADMINISTRATION OF C. PARVUM**
*Note that activated macrophages may themselves amplify or regulate the effects.*

by the earlier activation obtained in mice presensitised to C. parvum (Bomford and Christie, 1975). The apparent delay between the first appearance of cytotoxic peritoneal macrophages by day 2 or 3, and the development of activating cells in the spleen, could indicate that activation in vivo is mediated by another lymphocyte population such as that in the peritoneal cavity, and which responds at a different rate. However this explanation also has to account for the similar activation kinetics following i.p. or i.v. injection of C. parvum. Of relevance to this was the finding of Scott and Milas (1977) that although C. parvum was not detectable in peritoneal macrophages following i.v. administration, these cells were still cytotoxic, implying that activation either occurred outside the peritoneal cavity, or was mediated by sensitised lymphocytes migrating into it. An alternative explanation of the delay in appearance of activating cells may simply be that there were insufficient numbers of sensitised spleen cells before day 7 to be detectable in the activation assay.

Macrophage activation by splenic lymphocytes could be mediated by a factor, or factors released from the lymphocytes in the presence of C. parvum. It was not known whether activation in the presence of lymphocytes was also mediated by MAF. The nature of MAF was not investigated. It was thought possible that the C. parvum-mediated in vitro retention or regeneration of macrophage cytotoxicity may be caused by a regeneration of MAF, in the culture wells, by a small residual population of peritoneal lymphocytes. Macrophages could participate in MAF production although their involvement was excluded to a large extent by prior removal of glass adherent cells from

spleen cell suspensions. It would be interesting to know if activated macrophages could activate other non-cytotoxic macrophages. This may occur indirectly, anyway, through release and cleavage of complement products.

The relative roles of T cells and B cells in MAF production and macrophage activation have not been established unequivocally.

The above outline of possible macrophage activation models leaves many questions unanswered. For example, the question whether T cells or B cells, or both, are required for MAF release and macrophage activation ought to be investigated using homozygous nude mice and anti-B cell serum to supplement the methods used in this project.

In individuals with C. parvum-augmented specific tumour immunity (see Scott, 1975; Bomford, 1975) T cells presumably play an important part, either directly, or in cooperation with macrophages, in the expression of cytotoxic effects. It would be interesting to know if lymphocytes from such individuals could activate normal macrophages to express specific cytotoxicity, as in the SMAF system described by Evans and Alexander (1972a, b).

Another area of work which could be extended would be the investigation into C. parvum-MAF to discover its nature and its relationship both to lymphokines and to serum proteins such as albumin, and alpha-2-macroglobulin. Sephadex gel column separation of lymphocyte culture supernatants ought to provide some of the answers.

Experiments using bone marrow colony formation techniques would determine whether C. parvum can activate macrophage precursors directly, or through the complement-antibody system. The possible role of complement products in the mediation of C. parvum's biological

and anti-tumour effects ought also to be studied.

Finally, the possible role of natural killer cells as effectors of *C. parvum*'s *in vivo* and *in vitro* anti-tumour activity ought to be considered. It seems unlikely that the *in vitro* phenomena described in this thesis were mediated by such cells unless they displayed many of the physical and biological characteristics of macrophages such as adherence, phagocytosis, susceptibility to gold salt, and radioresistance. However, in the *in vivo* situation natural killer cells could be augmented by *C. parvum* treatment and thus play a significant role in anti-tumour effects. Confirmation of such an effect will probably have to await a suitable cell separation procedure.

## 9. CONCLUDING REMARKS

In conclusion, this thesis confirms that macrophage cytotoxicity can be induced *in vitro* by direct incubation, either with sensitised lymphocytes in the presence of *C. parvum*, or with culture supernatant containing lymphocyte-derived MAF. The results also affirm the necessity to administer *C. parvum* by the i.p. or i.v. routes in order to obtain a systemic anti-tumour effect. The subcutaneous route remains only of value for local effects.

Discounting the artificiality inherent in any *in vitro* model, it is likely that the activation system described is operative *in vivo* in the *C. parvum*-treated animal. This was supported by experiments assaying the *in vitro* cytotoxicity of PE cells from *C. parvum*-treated mice (Ghaffar and Cullen, 1977) which showed a similar pattern and time course of anti-tumour activity to that of the development of splenic

lymphocyte capacity to activate macrophages *in vitro*. Both these studies illustrated that the anti-tumour protection afforded by a single i.v. or i.p. injection of *C. parvum* declined with time. This decline may be akin to the loss of cytotoxicity of macrophages when cultured, and probably reflects the eventual elimination of *C. parvum* organisms from the body. This would prevent continued, new activation of macrophages as well as restimulation of lost macrophage activity.

In a clinical situation where it is desirable to sustain a maximal anti-tumour effect over a prolonged period, *C. parvum* would, with the possible exception of intratumour treatment, probably have to be administered at regular intervals. The parameters of dose, route, and timing of injections which would give optimal anti-tumour effects are still being evaluated. The high doses of *C. parvum* administered in the earlier animal studies had several undesirable consequences. Many T cell functions were suppressed (Scott, 1974c) with consequent increased risk of infection and the possible loss of the specific component of *C. parvum's* anti-tumour response. It should be noted that the dose of 1.4mg used in my experiments is now regarded as a large one and thus it would be interesting to repeat the activation experiments using smaller doses. High doses can also result in undesirable toxic effects (Scott, 1974c). Thus in animal studies *C. parvum* is now often given in "human equivalent" smaller doses. Scott and Warner (1976) demonstrated that weekly small doses of i.v. injected *C. parvum* gave an anti-tumour response as assessed *in vivo* and *in vitro* and had the added bonus of avoiding impairment of T cell function.

The nonspecific anti-tumour protection afforded by systemic _C. parvum_ injection is probably of particular importance to patients in the later stages of cancer who may have a depressed immune system. Possibly of greater value in immunocompetent patients would be the augmentation of a long-lasting specific response to their own tumour. This can be achieved by either injecting _C. parvum_ into, or close to, the tumour, or by injecting _C. parvum_ together with irradiated tumour cells subcutaneously. (Scott, 1975; Bomford, 1975). A successful outcome using the latter therapy requires use of the correct doses of _C. parvum_ and irradiated tumour cells (Bomford, 1975; Woodruff and Whitehead, 1977). Large doses of _C. parvum_ administered as a mixture with irradiated tumour cells abrogated the normal immunising effect of such irradiated tumour cells (Woodruff _et al_., 1976a). Recent work on this phenomenon points to the induction of specific immunological tolerance resulting from the injection of a large dose of _C. parvum_ mixed with irradiated tumour cells (Woodruff and Whitehead, 1977). Bomford (1977) has recently described further conditions which allow promotion rather than inhibition of the growth of MC-induced tumour cells in the footpads of _C. parvum_-treated mice. Promotion occurred with low doses of tumour cells (around the TD50), or with high doses of _C. parvum_ given before tumour challenge, except when i.v. _C. parvum_ was used in conjunction with very small numbers of tumour cells. Bomford argued that these findings were due, not to the presence of _C. parvum_-induced suppressor cells, but to the trapping of anti-tumour effector cells at the site of _C. parvum_ deposition. _C. parvum_ given before the tumour challenge would thus pre-empt the migration of such effector cells to the tumour site.

These studies demonstrate that considerable caution must be exercised when using C. parvum as a tumour immunotherapy agent and that further research into the mechanism of its action is required. Nevertheless, C. parvum, whether administered on its own or in conjunction with chemotherapy, radiotherapy or specific tumour immunotherapy, still promises to be of value in the treatment of residual or disseminated cancer. Just how promising it proves to be will depend on the outcome of the many current C. parvum clinical trials.

# APPENDIX

# APPENDIX

## Dulbecco's Phosphate Buffered Saline (PBS)

### Solution A (pH 7.3)

| | g./litre |
|---|---|
| NaCl | 8.00 |
| KCl | 0.20 |
| $Na_2HPO_4$ | 1.15 |
| $KH_2PO_4$ | 0.20 |

This formulation can be obtained in tablet form from Oxoid Ltd., London. Ten tablets are dissolved in 1 litre of distilled water and this solution is then autoclaved for 10 minutes at 115°C.

### Solution B

| | |
|---|---|
| $CaCl_2$ | 0.10 g./litre |
| $MgCl_2$ | 0.10 g./litre |

This can be obtained in 5ml sterile ampoules from Oxoid Ltd., London. Add 5 ml of solution B to 1 litre of solution A to make up the complete Dulbecco PBS of pH 7.4.

## Assessment of cell viability by trypan blue exclusion

The following method for the assessment of cell viability is based on that described by Waithe and Hirschorn (1973). 0.1 ml of cell suspension is added to 0.1 ml of 0.16 per cent (W/V) solution

of trypan blue in RPMI-1640. The suspension is then mixed and incubated for 5 minutes at 37°C. Viable (non-blue) cells are then counted immediately. The trypan blue assessment of viability was always compared with that obtained by phase-contrast microscopy in this study.

RPMI-1640 Medium (Gibco-Biocult Ltd., Paisley, U.K.)

| Vitamins | mg/litre | Amino Acids | mg/litre |
|---|---|---|---|
| Biotin | 0.2 | L-arginine | 200.0 |
| D-Ca pantothenate | 0.25 | L-asparagine $H_2O$ | 50.0 |
| Choline | 3.0 | L-aspartic acid | 20.0 |
| Folic acid | 1.0 | L-cystine | 50.0 |
| i-Inositol | 35.0 | L-glutamic acid | 20.0 |
| Nicotinamide | 1.0 | L-glutamine | 300.0 |
| Para-aminobenzoic acid | 1.0 | glycine | 10.0 |
| Pyridoxine HCL | 1.0 | L-histidine | 15.0 |
| Riboflavin | 0.2 | L-hydroxyproline | 20.0 |
| Thiamine HCL | 1.0 | L-isoleucine (Allo free) | 50.0 |
| Vitamin B12 | 0.005 | L-leucine (Methionine free) | 50.0 |
| Inorganic salts | | L-lysine. HCL | 40.0 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 100.0 | L-methionine | 15.0 |
| KCL | 400.0 | L-phenylalanine | 15.0 |
| $MgSO_4 \cdot 7H_2O$ | 100.0 | L-proline | 20.0 |
| NaCl | 6000.0 | L-serine | 30.0 |

| Inorganic salts (continued) | | Amino Acids (continued) | |
|---|---|---|---|
| $NaHCO_3$ | 850.0 | L-threonine (Allo free) | 20.0 |
| $Na_2HPO .7H_2O$ | 1512.0 | L-tryptophane | 5.0 |
| Other components | | L-tyrosine (Na salt) | 20.0 |
| Glucose | 2000.0 | L-valine | 20.0 |
| Glutathione (reduced) | 1.0 | | |
| Phenol red | 5.0 | | |

This is the formulation for RPMI-1640 medium as it appears in the Gibco-Biocult Ltd. catalogue. The medium is buffered with 20mM HEPES buffer.

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