THE ROLE OF MACROPHAGES IN THE NON-SPECIFIC STIMULATION OF THE ANTIBODY RESPONSE AS STUDIED IN VITRO

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by

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SUMMARY

The alteration of the function of macrophages in the primary antibody response from mice vaccinated or infected with various organisms was studied in the Marbrook culture system. In the first place the parameters of the system were investigated with respect to the response to two T cell dependent antigens (SRBC and DNP-KLH) and two T cell independent ones (DNP-POL and DNP-Ficoll). Then the antibody response to these antigens of spleen cells from mice pretreated with C. parvum or infected with M. lepraemurium, BCG, S. typhimurium and Plasmodium berghei yoelii was studied. An enhancement of the response to SRBC was found with cells from all pretreated mice, but in the case of M. lepraemurium and P. yoelii the effect was limited to the early stages of infection. No enhancement of the response to T independent antigens was found.

Spleen cells were separated into adherent and non-adherent cells by adherence to glass and the cell populations recombined in various ways. By this means, it was found that the adherent cells were responsible for the enhanced response. In the cases of C. parvum and M. lepraemurium pretreatment, this activation of adherent cells was shown to be dependent on the presence of T cells.

Spleen cells from later stages of M. lepraemurium infection showed a depressed response to SRBC which correlated with findings in vivo, while the response to DNP-POL was unaffected. This depression was also found to be mediated by macrophages, as was the depression of the SRBC response of spleen cells from mice infected with P. yoelii. However the response to DNP-Ficoll was also depressed in spleen cells from later stages of malaria infection. The mechanism of this suppression is not known.

The in vitro antibody response of spleen cells from animals pretreated with carrageenan was studied. It was found that pretreatment with carrageenan depressed the response to SRBC while not affecting the response to DNP-POL or DNP-Ficoll. Cell fractionation methods suggested

that the defect lay in the adherent cell population.

Using double Marbrook chambers it was confirmed that spleen cells from animals primed with KLH release a factor, when cultured in the presence of this antigen, that is capable of potentiating the in vitro response to DNP-KLH of unprimed spleen cells. Further work was done on the in vitro production and assay of this factor.

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LIST OF ABBREVIATIONS

B cell	bursa derived or equivalent lymphocyte
BCG	Bacille Calmette Guerin
CMI	cell mediated immunity
Con A	concanavalin A
C. parvum	*Corynebacterium parvum*
^{51}Cr-SRBC	chromium labelled sheep red blood cells
DNP	dinitrophenol
DNP-Fab	dinitrophenylated anti-SRBC Fab fragment
DNP-FG	dinitrophenylated fowl globulin
DNP-Ficoll	dinitrophenylated Ficoll
DNP-KLH	dinitrophenylated keyhole limpet haemocyanin
DNP-POL	dinitrophenylated polymerized flagellin
DNP-SRBC	dinitrophenol coated sheep red blood cells
Fab	antibody binding fragment
FGG	fowl gamma globulin
GRF	genetically related macrophage factor
GVH	graft versus host
IgG	immunoglobulin G
IgM	immunoglobulin M
KLH	keyhole limpet haemocyanin
LAF	lymphocyte activating factor
LPS	lipopolysaccharide
MIF	migration inhibition factor
2-ME	2 mercaptoethanol
Mlm	*Mycobacterium lepraemurium*
MLR	mixed lymphocyte reaction
MPS	mononuclear phagocyte system
PFC	plaque forming cells

PHA	phytohaemagglutinin
POL	polymerized flagellin
P. yoelii	*Plasmodium berghei yoelii*
SRBC	sheep red blood cells
SRF	skin reactive factor
T cell	thymus derived lymphocyte
TXB	thymectomized, X-irradiated, bone marrow reconstituted
XB	X-irradiated, bone marrow reconstituted

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CHAPTER ONE - GENERAL INTRODUCTION

Immunology as a science is a young one, arising from bacteriology as the study of the host's acquired antimicrobial response to infectious pathogenic organisms. Early in the developement of immunology, there was much argument as to which was the more important defence mechanism - that of humoral antibody or that of phagocytic cells (Metchnikoff, 1905). When it was discovered that phagocytosis could be enhanced as a result of serum antibodies specific to the invading organism (the process now known as opsonization) a link between the two aspects of the immune response was made. The field of immunology has expanded widely since then and much evidence has been amassed for the interaction and synergism between cell-mediated immune responses and humoral immunity. Cell-mediated immunity is usually defined as any immune response that can be transferred by cells and includes such phenomena as allograft rejection, graft versus host disease and delayed hypersensitivity. Humoral immunity is defined as immunological reactions transferable by serum and includes immediate hypersensitivity or allergic responses and Arthus reactions as well as classical antibody mediated protection. As well as realizing that there are two distinct aspects of the immune response, it is now well understood that there are two distinct types of lymphocyte which mediate them.

Evidence for two distinct lymphocyte types

This evidence comes from two main sources. Glick et al. (1956) reported that the removal of the bursa of Fabricius, a cloacal lymphoid organ, from chickens impaired their ability to produce serum antibodies following subsequent antigenic challenge. This was confirmed using hormonal inhibition of bursal developement (Mueller et al., 1960) and by Warner et al. (1962) who also showed that bursectomy had no effect on homograft or graft versus host reactions. Further studies (Janković et al., 1963; Cooper et al., 1966) showed that thymectomy at hatching depressed delayed hypersensitivity responses. Cooper and his co-workers (1966) provided compelling evidence for the existence of two distinct compartments of the immune system of the chicken. Neonatal thymectomy was shown

to impair homograft rejection in mice (Miller, 1961, 1962) and rats (Arnason et al., 1962), delayed hypersensitivity in rats (Jankovic et al., 1962) and have a variable effect on antibody responses in mice (Miller, 1962; Good et al., 1962). Thymectomy was accompanied by lymphopenia which however only depleted certain areas of lymphoid tissue (Parrot et al., 1966) while other zones containing follicles, germinal centres and plasma cells were unaffected. These observations suggested a subdivision of the immune response and also that the peripheral lymphoid tissues contained two distinct populations of cells.

Further evidence for two lymphocyte types comes from the study of immunodeficiency states in man. Good and his co-workers have defined a spectrum of immunological deficiency diseases. At one end is Bruton-type agammaglobulinaemia (sex-linked) with no antibody responses but intact cell-mediated immunity, and at the other extreme is Di George's syndrome in which cell-mediated immunity is absent and antibody production is only moderately affected (reviewed by Good et al., 1971).

Cell co-operation

The two lymphocyte classes have been termed T cells for thymus derived lymphocytes responsible for cell-mediated immunity and B cells, for bursal derived or equivalent cells, responsible for antibody production. There is now plenty of evidence for the co-operation between T and B cells in the response to certain antigens. The first suggestion of this was the work of Claman et al. (1966) in which they found that irradiated mice given both thymus and bone marrow cells made a far greater primary response to SRBC than recipients of either thymus or bone marrow cells alone. Miller and Mitchell (1968) found that neonatally thymectomized mice which failed to give a response (humoral) to SRBC could be restored with semiallogeneic thymus cells or thoracic duct cells. By using alloantisera, they demonstrated that the antibody forming cells were recipient in origin and therefore probably derived from B cells (Mitchell and Miller, 1968). Work using protein hapten carrier molecules has also

contributed to the understanding of cell co-operation. It was found that mice would respond to a hapten when coupled to a protein carrier and Ovary and Benacerraf (1963) showed that rabbits primed with DNP-bovine gamma globulin (DNP-BGG) produced a secondary response only if the secondary immunization was with DNP on the same carrier. Rajewsky et al. (1969) and Mitchison (1969, 1971) both showed that it was possible to overcome this carrier effect if the responding animal was previously primed to the heterologous carrier. Thus they suggested that there was a population of cells reactive to the carrier and a population of cells reactive to the hapten. There is now evidence that the carrier responsive cells are the T cells and that the hapten responsive cells are the B cells. The most convincing evidence for this is that helper cells can be inhibited by anti-theta serum and complement, while hapten primed cells are not (Raff, 1970). Not all antigens require T-B cell co-operation for the humoral response and these will be discussed later in the introduction.

There is both direct and indirect evidence to establish a role for macrophage-lymphocyte interaction in the induction of immune responses, but before this is discussed the general properties of the macrophage will be defined.

General properties of the macrophage

Metchnikoff was the first person to attempt a classification of phagocytic cells (Metchnikoff, 1892). He divided them into two groups - the macrophages and the microphages (smaller phagocytic cells generally polymorphonuclear cells). He showed that both these cell types were capable of phagocytosis and played an important role in the host resistance to infection. It was he who realized the connection between mononuclear phagocytes in the spleen, lymph nodes and bone marrow and those outside these organs and he introduced the term "the macrophage system". Aschoff in 1924 grouped these cells into what he called the reticuloendothelial system (RES). His system of classification has been much criticised as many of the criteria on which he based his classification

could apply to more than one cell type e.g. the uptake of vital dye. Even poorly phagocytic cells such as endothelial cells can become labelled due to pinocytosis when large amounts of dye are applied. Therefore during a conference on mononuclear phagocytes in 1969 a new classification was proposed. All highly phagocytic mononuclear cells and their precursors on the basis of morphology, function and kinetics were placed in one system, the mononuclear phagocyte system (MPS). The functional criteria of this system are:-

1. Avid phagocytosis and pinocytosis and the ability to adhere to glass (Rabinovitch, 1968, 1970; Cohn, 1968, 1970).

2. Cell surface receptors for immunoglobluin and complement (Berken and Benacerraf, 1966; Lay and Nussenzweig, 1968, 1969; Nelson, 1969; Huber and Fudenberg, 1970).

3. Various morphological criteria - shape of nucleus, nature of surface membrane etc.

4. Cytokinetic studies (van Furth, 1970a, 1970b; Spector and Ryan, 1970). These studies showed that mononuclear phagocytes originate from precursor cells in the bone marrow, are transported via the peripheral blood as monocytes and eventually become tissue macrophages.

Role of macrophages in immunological reactions

Since Metchnikoff's first observations of the macrophage its role in the immune response has been controversial. In the 1920's macrophages themselves were thought to be responsible for both the uptake of antigen and production of antibody (Sabin, 1923). However the developement of cellular technology has enabled a much greater understanding of the system. One particular difficulty in studies on macrophage involvement in immune responses is the lack of precise definition distinguishing these cells and their precursors from lymphocytes (reviewed by Cohn, 1968; van Furth, 1970b). Functional tests may also be misleading since macrophages and lymphocytes can bind cytophilic antibody (Coulson et al., 1967) under the right conditions and lymphocytes can also adhere to glass or

cotton (Shortman et al., 1971). Anti-macrophage serum has been used in in vivo studies but there is no unequivocal evidence that it does not cross react with lymphocytes (Schwartz et al., 1970; Shortman and Palmer, 1971). It is also very difficult to remove all the macrophages from any cell population and it is possible that a very few adherent cells are needed for the production of a response to either antigen or mitogens.

Evidence for macrophage involvement in the immune response comes from various sources:-

1. Morphological - The formation of clusters of macrophages and lymphocytes both in tissue sections and in tissue culture (reviewed by Schwartz et al., 1970).

2. Functional

(a) The immune response in vitro - The removal of macrophages from lymphocyte suspensions by methods such as adherence, phagocytosis or anti-macrophage serum abrogates the response to a variety of antigens (Mosier and Coppleson, 1968; Feldmann and Palmer, 1971).

(b) Immunogenicity of macrophage bound antigen (reviewed by Unanue and Cerottini, 1970). All antigens so far tested are immunogenic when transferred on or in macrophages and some, but not all, show increased immunogenicity.

(c) Fate of antigen taken up by macrophages (see later).

The role of the macrophage as understood in the light of current knowledge will be discussed for the rest of this introduction.

T lymphocyte activation

In vitro activation of primed T cells by soluble antigens is macrophage dependent. This has been demonstrated by methods such as removal of adherent cells (Oppenheim et al., 1968), inactivation of phagocytic cells with thorotrast (Oppenheim et al., 1966) or carrageenan (Lake et al., 1971). The depleted immune lymphocytes could be restored by macrophages of various types, e.g. immune or nonimmune, peritoneal or alveolar

(Seeger and Oppenheim, 1970). Very few macrophages are needed to demonstrate significant blastogenesis in these systems, less than 0.1% in cultures of certain guinea pig lymphoid cell populations (Rosenstreich and Rosenthal, 1973).

T cell activation by allogeneic antigens requires macrophages. However, the mixed lymphocyte reaction (MLR) is a primary response as compared to the response to soluble antigens which is a secondary response. In both humans (Alter and Bach, 1970) and mice (Phillips et al., 1972), the MLR has been found to be macrophage dependent. Macrophages are also required for the induction in vitro of cytotoxic T cells i.e. cell-mediated lympholysis (Wagner et al., 1972).

Whether macrophages are required or not for the mitogen induced T cell proliferation is controversial. Studies using high PHA concentrations and high cell concentrations suggested that the response was macrophage independent (Jehn et al., 1970). However the response to suboptimal doses was enhanced by the addition of macrophages (Oppenheim et al., 1966). Recent findings showed that the response to PHA of purified human T cells was enhanced by macrophages but that at high mitogen concentration there was a small response in T cells alone (Lohrman et al., 1974). Two points should be borne in mind, firstly the difficulty of removing all macrophages from a system, and secondly that T cell subpopulations may vary in their macrophage dependency.

B cell activation

This is more difficult to ascertain as both macrophages and B cells are adherent which means that it is difficult to obtain macrophage free B cells. It is generally accepted, however, that macrophages are more adherent than B cells and that macrophage rich cell populations can be obtained by adherence to glass. Also, if other criteria, such as sensitivity to X-irradiation, are applied then it is possible to differentiate more fully between B cells and macrophages. In addition, as many means of activating T cells are macrophage dependent, any B cell response to a

thymus dependent stimulant would automatically be macrophage dependent. Therefore in many cases B cell activation may indirectly require macrophages.

Thymus dependent antigens

The primary response to SRBC is macrophage dependent (Mosier, 1967) as shown by the fact that the cell required to restore the response of nonadherent cells is:-

1. radioresistant (Roseman, 1969; Sjöberg et al., 1972)
2. phagocytic (Shortman et al., 1970)
3. sensitive to anti-macrophage serum (Shortman and Palmer, 1971)
4. present in macrophage rich peritoneal cells (Hartmann et al., 1970).

The macrophage requirement for the secondary response is more unclear. Some reports (Pierce, 1969) claim that it is macrophage independent, others say it is macrophage dependent (Feldmann and Palmer, 1971). The general consensus of opinion would appear to be that macrophages are needed for the secondary response but for shorter periods of time and at a lower concentration.

The requirement for macrophages for the response to soluble thymus dependent antigens such as DNP-Fowl globulin (DNP-FG) or DNP-keyhole limpet haemocyanin (DNP-KLH) is also disputed. Feldmann (1972) has shown that the response to DNP-FG of primed spleen cells can be abrogated by the removal of macrophages. However, Katz and Unanue (1973) found the response to DNP-KLH of primed spleen cells was not affected by the removal of adherent cells. Macrophages have been shown to overcome the suppressive effects of excess helper T cells (Feldmann, 1974).

There are various reports that the addition of 2-mercaptoethanol can completely replace the need for adherent cells in the in vitro anti-SRBC response (Chen and Hirsch, 1972a). They suggested that the primary action of 2-ME on non-adherent cells was in promoting their viability and reactivity (Chen and Hirsch, 1972b).

Thymus independent antigens and mitogens

The response to antigens such as polymerized flagellin (POL) and DNP-POL and mitogens such as endotoxin are macrophage independent *in vitro* (Feldmann and Palmer, 1971; Yoshinaga *et al.*, 1972). Also, thymus dependent antigens can be made thymus and macrophage independent by altering the size or spatial configurations. Solubilized SRBC are both T cell and macrophage independent (Feldmann and Palmer, 1971). TNP-KLH can be made T cell and macrophage independent by coupling to Sepharose beads (Feldmann *et al.*, 1974). However, Lee *et al.*, (1976) report that POL, DNP-Ficoll and soluble SRBC are dependent on a macrophage accessory cell.

Role of macrophages in processing and presenting antigens to lymphocytes

The role of macrophages in processing and presenting antigen *in vivo* has been discussed at length by Unanue (1972). Macrophages take up antigen by phagocytosis, pinocytosis and passive adsorbtion.

Most antigenic material once bound to macrophages is degraded to a nonimmunogenic form (Ehrenreich and Cohn, 1967) but approximately 10% is retained in an immunogenic form and can remain so for up to 7 days (Unanue and Cerottini, 1970). Once the antigen is bound to the macrophages it can activate lymphocytes even when the macrophages are subjected to X-irradiation; mitomycin C or actinomycin D (Rosenstreich and Rosenthal, 1973; Rosenthal *et al.*, 1973; Seeger and Oppenheim, 1970).

The cellular localization of immunogenic antigen is uncertain. Unanue and Cerottini (1971) presented evidence that it remained on the plasma membrane. However, Calderon and Unanue (1974) showed that some of the membrane bound KLH was first internalized and then slowly released into the culture medium.

The handling of SRBC by macrophages is as yet not clearly understood. Macrophages are able to bind and present SRBC in an immunogenic form both *in vivo* and *in vitro*, though only surface bound SRBC are immunogenic. Unanue (1972) found that macrophage bound SRBC was less immunogenic *in*

vivo than an equivalent amount of free SRBC.

Antigen presentation is also debated. There is evidence both for and against the need for direct macrophage-lymphocyte contact and also for the requirement for histocompatible macrophages.

Several groups of workers have produced evidence for the need for direct cell to cell contact. Observations have been made that lymphocytes and blast cells form clusters around macrophages (Mosier, 1969). Also, no stimulation occurs if macrophages and lymphocytes are separated by millipore filters (Hersh and Harris, 1968). Evidence against direct cell to cell contact comes from several groups. McIntyre and Pierce (1973) showed that if cell clusters and single lymphocytes from mouse spleen cell cultures were separated, the clusters produced very few PFCs, while the single lymphocytes produced many PFCs. Other workers (Calkins and Golub, 1972) showed that a primary response to SRBC could occur even if B cells were separated from macrophages by millipore filters.

Recently, Rosenthal and Shevach (1973) showed that the proliferative response of guinea pig lymphocytes to antigen had a requirement for histocompatible macrophages. Macrophages did not stimulate lymphocytes of a different histocompatibility, though in a histocompatible environment, these same macrophages were capable of stimulating lymphocytes. Other studies have not shown this absolute requirement for histocompatible macrophages and lymphocytes (Cosenza and Leserman, 1972).

The continuining work of Feldmann and his co-workers has produced data relevant to both the possible need for cell to cell contact between macrophages and lymphocytes and the requirement or otherwise for histocompatible macrophages.

Feldmann and Basten (1972), using double Marbrook vessels, showed that it was possible to induce specific collaboration across a cell impermeable membrane. They were also able to demonstrate the production of a factor from activated T cells in the upper chamber which was able to induce a response in the B cell/macrophage compartment below. This they

called IgT and it has been suggested that it is cytophilic for macrophages. Further work by Feldmann and his co-workers demonstrated that macrophages are required for the generation of helper T cells in vitro (Erb and Feldmann, 1975a). Direct contact was not needed between the macrophages and the T cells, and the factors released from macrophages were as effective in activating T cells as the macrophages themselves (Erb and Feldmann, 1975b). They found two different types of factor:-

1. Supernatants from purified macrophages incubated with antigen for several days generated helper cells in the absence of macrophages or additional antigen, but only if the macrophages and T cells were identical at the I-A subregion of the H-2 complex. This, they designated genetically related macrophage factor (GRF).

2. Supernatants obtained from macrophages incubated for several days without antigen replaced macrophages only if the antigen is particulate. This factor was not genetically restricted so they called it nonspecific macrophage factor.

Other factors produced by macrophages

Early reports by Dutton et al. (1970) and Hoffman and Dutton (1971) showed that supernatants from peritoneal macrophages cultured with SRBC allowed nonadherent spleen cells to respond to SRBC. This factor is suggested to act by solubilizing antigen from SRBC and so making it immunogenic for lymphocytes in the absence of macrophages. Bach et al. (1970) also reported that macrophage supernatants can allow purified nonadherent spleen cells to respond to allogeneic cells in the MLR. Gery and Waksman (1972) reported that macrophages produce a substance called lymphocyte activating factor (LAF) that enhanced the proliferation of T cells to PHA and Con A. The nature of the action of this factor is not known. Schrader (1973) reported that peritoneal cells release a factor into the culture medium which facilitated the activation of B cells to fowl IgG (FGG). Recently, Calderon and Unanue have reported two biological activities regulating cell proliferation in cultures of peritoneal

exudate cells. One exerted an inhibitory and the other a stimulatory effect on spleen cell and thymocyte proliferation. The stimulating supernatant also allowed spleen cells from nude mice to make a PFC response to SRBC of both the IgM and IgG classes (Calderon et al., 1975).

The suppressive effect of macrophages on lymphocytes

One of the first reports of macrophages suppressing lymphocyte activity was by Parkhouse and Dutton (1966). They showed that the addition of more than 5% alveolar or peritoneal exudate macrophages suppressed the proliferative response of rabbit spleen cells to PHA or antigen. This suppression could not be mediated by supernatants but only by direct cell to cell contact. Macrophages activated by means of a graft versus host (GVH) reaction have been shown to depress the response to both a thymus dependent and a thymus independent antigen (Sjöberg, 1972). Scott (1972a, b) showed that adherent cells from C. parvum pretreated animals were inhibitory to both PHA and MLR responses. Work by Waksman and his coworkers (Yoshinaga et al., 1972) showed that suppression macrophages were of two kinds, firstly one that suppresses at higher concentrations (5% or more) and was associated with typical macrophages and secondly a suppressor cell that is found in mouse spleen cell populations after activation by means of GVH or C. parvum. This suppressor cell is adherent, phagocytic and suppresses T as well as B cell responses. For a while the exact nature of this suppressor cell was unclear but there is now evidence in the rat system that this cell is an adherent T cell (Folch and Waksman, 1973a,b). Macrophages can suppress the response to thymus independent antigens, as in the case of DNP-Ficoll (Mosier et al., 1974) in the mouse.

There have been various reports of the production of suppressive factors by macrophages. Supernatants from endotoxin-activated mouse peritoneal macrophages suppressed the response of spleen cells to PHA, Con A, endotoxin and to allogeneic cells (Nelson, 1973). A suppressor activity has also been demonstrated in supernatants from oil induced peritoneal cells of the rat that had been cultured for 24 hours (Waldman and Gottlieb, 1973).

Activation of macrophages

There is plentiful evidence that macrophages play a role in the resistance to infections either as described by Metchnikoff (1905) with organisms which are readily killed by macrophages and in which antibody can enhance ingestion by opsonization, or, with organisms which can survive and multiply within macrophages. Here antibody has no role and activated macrophages are essential (Lurie, 1964; Mackaness, 1964). These usually come from animals that have suffered a mild infection. Recent in vivo studies suggest that immune activation of macrophages requires T cells sensitised to the infecting agent (Lane and Unanue, 1972; Mackaness, 1969). The mechanism of in vivo macrophage activation is not known but in vitro studies have served to produce a model of the possible mode of activation. These studies have shown that stimulated lymphocytes produce a number of soluble factors (Bloom and Bennett, 1966; David, 1966; David and David, 1972; Dumonde et al., 1969).

These soluble factors can be produced by culturing guinea pig lymph node cells from immune animals in the presence of specific antigen. The supernatants of these cultures are chromatographed on Sephadex columns and fractions rich in migration inhibition factor (MIF) are added to macrophage monolayers (Remold et al., 1970, 1972). Macrophages cultured thus for 3 days show a number of changes such as - enhanced ability to adhere to glass, enhanced mobility and spreading (Nathan et al., 1971), increased phagocytosis of some particles and decreased phagocytosis of others (Remold and Mednis, 1972), increased glycolytic activity (Nathan et al., 1971), an apparent loss of certain lysosomal enzymes with an increase in cytoplasmic granules (Remold and Mednis, 1972). Macrophages activated by means of incubation in MIF rich fractions also show an increased bacteriostasis (Fowles et al., 1973) and an enhanced cytotoxicity for tumour cells (Piessens et al., 1974). It is also possible to activate macrophages by the direct presence of lymphocytes and antigen rather than through the agency of lymphocyte mediators. This activation differs in

only needing a 24 hour culture period before the macrophages begin to show characteristic signs of activation in comparison to the 3 days needed when using soluble mediators (Simon and Sheagren, 1971). There is evidence that it is T cells which are involved in this in vitro activation (Krahenbuhl et al., 1973).

Role of macrophages in resistance

(a) Infection

As previously stated, protection from the harmful effects of pathogenic bacteria and viruses is a central function of the immune system, both CMI and humoral immunity being involved. It is probably the interactions between T cells, B cells and macrophages and their soluble products that govern the outcome of an infection (World Health Organisation, 1973).

Mackaness (reviewed 1971) has clearly demonstrated the central importance of macrophages in the resistance to intracellular facultative organisms e.g. Listeria, Brucella, Salmonella and tubercule bacilli. An important feature of this antimicrobial protection is its transient non-specific effect. Animals sensitized with M. tuberculosis have an enhanced resistance to antigenically unrelated organisms such as Listeria monocytogenes. There are two important features of this system:-

1. Immunity is transferable by lymphocytes, but not by macrophages or serum. Anti-theta serum and complement treatment of lymphocytes abolishes their ability to transfer immunity suggesting that T cells are important (Lane and Unanue, 1972).

2. Successful transfer of immunity with lymphocytes is dependent on the integrity of the recipient's MPS. Therefore T cells provide a specific and macrophages a non-specific mechanism (Mackaness, 1971a,b) in resistance to infection.

The basis of this interaction probably consists of the nonspecific activation by lymphocyte products e.g. MIF which are released by sensitized lymphocytes in response to antigen stimulation (McGregor and Koster, 1971).

Blanden (1971) analysed the cell types involved in protection against ectromelia virus and showed that theta positive cells were necessary and probably responsible for initiating recovery from this infection and that nonspecific radiosensitive monocytic cells probably made the major contribution to the effector mechanism. Salvin (1972) has recently summarized evidence suggesting that CMI is the principal defence mechanism against many systemic fungal infections. The close relationship between cell-mediated immunity to infection and classical delayed hypersensitivity reactions has been pointed out by Mackaness (1971b) and there is in fact evidence for T cell-monocyte/macrophage interaction at the inflammatory sites of delayed or "contact" sensitivity reactions (Williams and Waksman, 1969; Tubergen and Feldman, 1971).

(b) Macrophages and mycobacteria

Mycobacteria are capable of growth inside cells and in the case of M. leprae (the human leprosy bacillus) and M. lepraemurium they are obligate intracellular parasites growing in the cells of the MPS. As they do not produce toxins, humoral responses play no part in the defence against them (Mackaness, 1968). Resistance to mycobacterial infections therefore depends on CMI and the production of sensitized T cells, soluble factors and activated macrophages. The T cells provide a specific mechanism whereas macrophages are nonspecific mechanism (Mackaness, 1971a,b). There are reports suggesting that macrophage activation is a local phenomenon (Dannenberg et al., 1968) only observed systemically in experimental conditions which produce an extreme degree of stimulation (Mackaness, 1968; Blanden et al., 1969).

(c) Macrophages and tumours

In recent years the main focus of interest in tumour immunology has moved from problems such as the possession or otherwise of tumour specific antigens by malignant cells to the problem of why the immune system is so ineffective at removing/rejecting tumours. Results of these studies suggest that there are a wide variety of ways in which tumours

may be rejected. With reference to macrophages it has been shown that adherent cells from immunized animals can respond to the sensitizing antigen (tumour cells) by a cytotoxic reaction against a variety of unrelated target cells *in vitro* (Granger and Weiser, 1964; Evans and Alexander, 1972; Lohmann-Matthes *et al.*, 1972). Evans and Alexander suggest that the activation of macrophages is a two stage process - first, the macrophages are specifically armed and can only kill cells to which they have been sensitized and then they are activated and become non-specific in their cytotoxicity. They have evidence that suggests the need for T cells in the specific arming (Evans *et al.*, 1972). This activation of macrophages may result from the uptake of cytophilic antibody or by the action of lymphocyte products (MIF etc.) on macrophages. Macrophage cytotoxic activity can also be elicited nonspecifically by double-stranded RNA, Lipid A and endotoxin (Alexander and Evans, 1971) and as a result of Toxoplasma infection (Hibbs *et al.*, 1972).

Therefore the role of the macrophage in the immune response is very complex and with this in mind it was attempted to develop an *in vitro* system in which the need for macrophages could be demonstrated and then to assess their role in the immune response. The system decided on was the *in vitro* antibody synthesis system of Marbrook (1967). Using this system, it was decided to investigate the effect of the adjuvant *C. parvum*, infections such as BCG, *S. typhimurium*, and malaria and carrageenan on the *in vitro* antibody response.

CHAPTER TWO - MATERIALS AND METHODS

A. MATERIALS

1. Animals

1:1 Inbred CBA female mice aged between 12 to 16 weeks were used in most experiments. Their weights ranged from 15 to 23 g. They were bred in the Medical School animal house from stock originating from the M.R.C. Radiobiology Unit, Harwell, England. Ex-breeder CBA females were used in a few experiments and their use will be indicated in the text as appropriate.

1:2 Randomly bred white albino Wright-Fleming mice were used to maintain the Mycobacterium lepraemurium infection. These mice are peculiar to this institute.

1:3 Inbred C3H female mice aged between 12 to 24 weeks were used in a few experiments. These mice were bred in the animal house from stock originating from NIH, Bethesda, U.S.A.

1:4 Inbred C57/Bl female mice were used in a few experiments. These mice were aged between 16 to 24 weeks and were bred in the animal house from stock originating from the M.R.C. Radiobiology Unit, Harwell, England.

2. Culture Media

RPMI 1640 was obtained bottled in powder form (sufficient to make 50 litres of medium) from Flow Laboratories, Scotland. This powder contained glutamine but no sodium bicarbonate. RPMI 1640 powder was added, at a concentration of 10.43 g/l, to double distilled water, at a volume 5% less than the desired final volume, together with 2.0 g/l sodium bicarbonate (Analytical Reagent Grade); 3.5 g/l HEPES (Hopkin and Williams, England) and the antibiotics cloxacillin and ampicillin (Beecham Laboratories, England) at a concentration of 125 mg/l and gentamicin (Nicholas Laboratories Ltd., England) at a concentration of 10,000 I.U./l. Prior to their addition, ampicillin and cloxacillin were both made up in 1.5 ml of sterile double distilled water. The solution was stirred until almost

all the solids were dissolved, as there was always a small residue of the RPMI 1640 powder that never dissolved completely. The volume of the medium was adjusted to the desired volume with double distilled water and the pH adjusted to 7.2 with either 1N sodium hydroxide or 1N hydrochloric acid. The medium was sterilized by filtration through Sartorius Membrane Sterilization Filters, pore size 0.2 μ, and was stored in sterile 400 and 500 ml bottles until needed at 4°C. For cell cultures, foetal calf serum was added at a final concentration of 5%.

3. Foetal calf serum (FCS)

This was supplied by Flow Laboratories, Scotland. Each time a new batch was required, samples were tested for their ability to support in vitro antibody synthesis. All foetal calf serum was heat inactivated at 56°C for 30 minutes before use. It was stored in 100 ml aliquots at -20°C.

4. Physiological saline

A 0.9% w/v solution of sodium chloride (Analytical Reagent Grade) was prepared in distilled water. It was sterilized by autoclaving at 120°C and 15 psi for 15 minutes.

5. Hibitane

0.5% chlorhexidine in 70% methylated spirit was obtained from the pharmacy of St. Mary's Hospital, London.

6. Anaesthetic

Avertin (Winthrop, Surrey, England) was used to lightly anaesthetise mice before thymectomy. A working solution was produced by diluting 0.3 ml Avertin with 20 ml warm sterile saline and this was injected intraperitoneally, giving 0.01 ml Avertin per gram body weight.

7. Antigens

7:1 Sheep red blood cells (SRBC) in Alsever's solution were supplied by Tissue Culture Services Ltd., Slough, England. They were washed three times in sterile saline before use.

In vivo - mice were injected intravenously, via the lateral tail vein. The dose was either 5×10^6 or 10^9 SRBC in 0.2 ml saline.

In vitro - SRBC were generally added to cultures at a final concentration of 2×10^6 SRBC/culture. In early experiments other concentrations were used and these will be discussed in the text as appropriate.

7:2 Di-nitrophenylated polymerized flagellin (DNP-POL) was a kind gift from Dr. Marc Feldmann.

DNP-POL was usually added to cultures at a final concentration of 0.1 µg/culture. However in certain experiments concentrations ranging from 0.001 µg to 1 µg/culture were used. This will be indicated in the text as appropriate.

7:3 Di-nitrophenylated keyhole limpet haemocyanin (DNP-KLH) was a kind gift from Dr. A. Boylston. It was normally added to cultures at a final concentration of 1 µg/culture. In certain experiments other concentrations were used and these will be discussed in the text as appropriate.

7:4 Di-nitrophenylated Ficoll (DNP-Ficoll) was a kind gift from Dr. A. Boylston and was normally added to cultures at a concentration of 0.02 µg/culture. In certain experiments other concentrations were used and these will be discussed in the text as appropriate.

7:5 Keyhole limpet haemocyanin (KLH) was obtained from the Sigma Chemical Company, St. Louis, U.S.A.

Mice aged 16 to 20 weeks were primed to KLH by injecting 100 µg KLH and 100 µg freeze-dried human tubercule bacilli mixed with 0.2 ml Alhydrogel (Miles Seraval (Pty) Ltd., Maidenhead, Berks., England). Ground human tubercule bacilli at a concentration of 2 mg/ml in phosphate buffered saline were mixed with an equal volume of a suspension of KLH also at a concentration of 2 mg/ml and incubated for one hour before the addition

of Alhydrogel. The animals were given two injections of 0.3 ml intraperitoneally ten weeks apart. They were used at least 6 weeks after the second injection.

7:6 Antigens used in the Cunningham assay

7:6:1 A 20% suspension of SRBC was used to assay the response to SRBC.

7:6:2 DNP-coated SRBC (DNP-SRBC)

To assay the response to DNP-POL, DNP-KLH and DNP-Ficoll, 10% washed SRBC were incubated with a dinitrophenylated anti-SRBC Fab fragment (DNP-Fab) for 30 minutes at 37°C. The DNP-coated SRBC were washed 2 or 3 times with saline and used as a 10% solution.

The DNP-Fab was a gift from Dr. Marc Feldmann and the method of production of DNP-SRBC was that of Strausbach et al. (1970).

8. Antibiotics

Mice that had been irradiated were given antibiotics to minimise infection and subsequent death. Their preparation was as follows:- 1,000,000 I.U. Colomycin (Colistin sulphomethate B.P.) was dissolved in 40 ml of sterile saline and into this 500 mg ampicillin (Beecham Labs., England) was dissolved. The dose given was 0.2 ml (2.5 mg ampicillin and 5,000 I.U. colomycin)(Šljivić, 1970). This dose was injected subcutaneouxly twice daily for 10 to 14 days, depending on the appearance of the mice.

9. Non-specific stimulants

9:1 Corynebacterium parvum (C. parvum)

9:1:1 In vivo - Mice were injected intravenously with 0.2 ml of a 1 in 4 dilution of the standard suspension containing 7 mg/ml dry weight of formalin-killed C. parvum organisms (Wellcome Research Laboratories, Beckenham, Kent, England; Batch PX289).

9:1:2 In vitro - C. parvum CN6134 (Batch PX365B) containing 7 mg/ml dry weight of C. parvum organisms, a gift from Dr. C. Adlam, Wellcome

Research Laboratories, was used for *in vitro* studies. These were heat-killed organisms containing no preservative.

9:2 Carrageenan

Sea Kem 9, carrageenan (REX 7220) was a gift from Marine Colloids Inc. It was suspended in physiological saline before use at a concentration of 10 mg/ml. The animals received either 1 injection or 4 injections every other day, of 0.1 ml intraperitoneally.

10. Infections

10:1 Malaria - mice were infected with *Plasmodium berghei yoelii*. Further details are given later in this section.

10:2 Bacille Calmette Guerin (BCG) was obtained from Glaxo Laboratories as a dried vaccine in 10 ml or 1 ml ampoules which were reconstituted with saline. Further details are given later in this section.

10:3 *Salmonella typhimurium strain C5* was originally from Professor D. Rowley, Adelaide, Australia. Its LD_{50} for CBA mice was 1×10^7 organisms and it was maintained on Dorset Egg Agar slopes at $4^{\circ}C$. Further details are given later in this section.

11. Marbrook culture vessels

11:1 Single chamber vessels

The *in vitro* antibody synthesis system used was the Marbrook system (1967). The outer vessel was a glass Universal container and the inner vessel was a glass tube, internal diameter 12 mm, with a hole blown in the side to allow more rapid equilibration of conditions in the two vessels. The inner vessel was held in place with a ring of silicone rubber tubing, internal diameter 14 mm, and between the tubing and the inner vessel was a $1\frac{1}{2}$ inch gauge 19 hypodermic needle (Sherwood Medical Industries Ltd.). This needle was present to facilitate equilibration of conditions between the outer vessel and the atmosphere in the incubator. The upper end of the inner vessel was plugged with non-absorbent cotton

DIAGRAMS 1A AND 1B -MARBROOK VESSELS

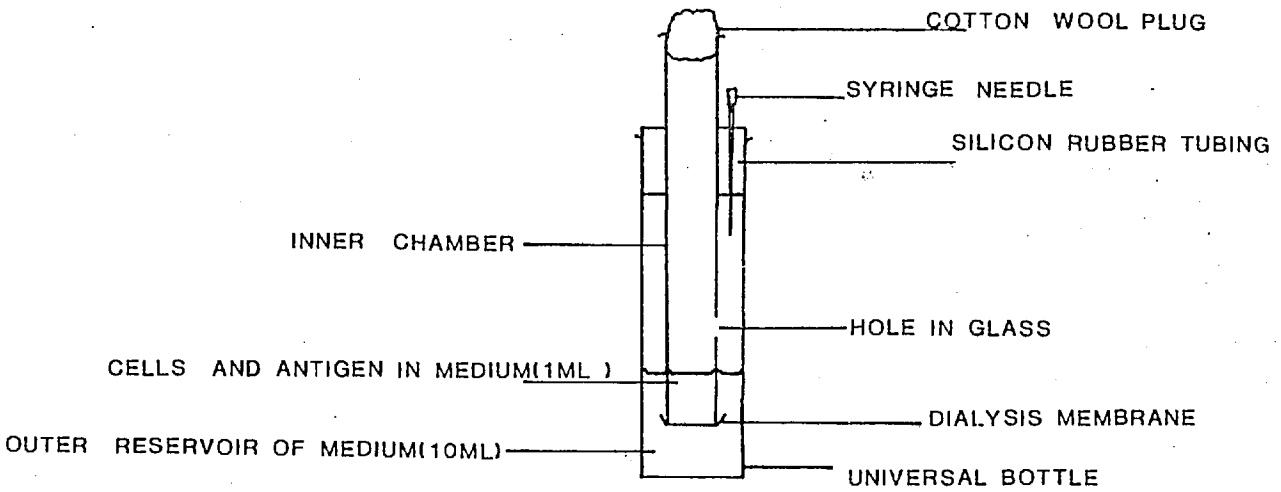


DIAGRAM 1A -SINGLE MARBROOK VESSEL

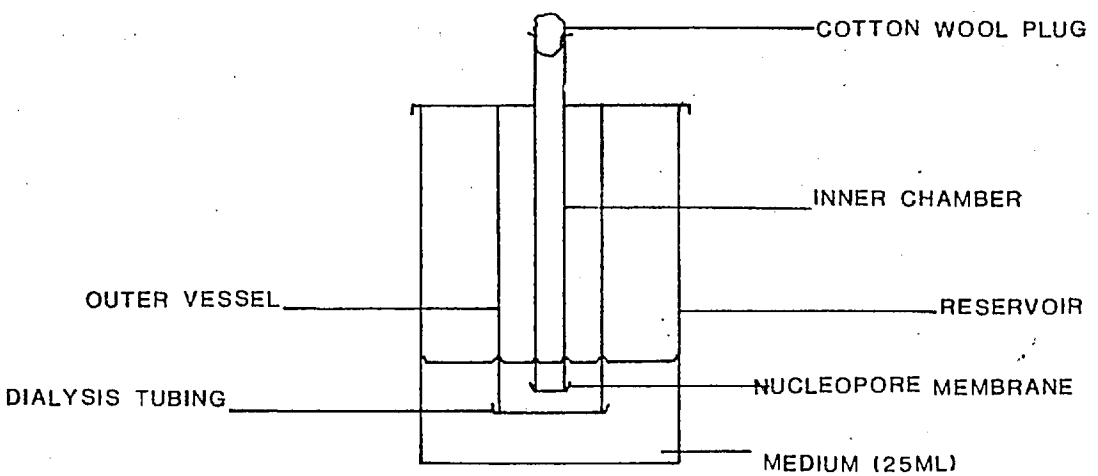


DIAGRAM 1B -DOUBLE MARBROOK VESSEL

wool. The lower end of the inner vessel was covered with dialysis membrane (The Scientific Instrument Centre Ltd., London) which had been boiled twice in distilled water as had the rubber bands (size 6 - H.A. Coombs Ltd., Wiltshire, England) which held the dialysis membrane in place. The vessels were sterilized by autoclaving at 115°C:10 psi for 15 minutes. For further details of the culture vessel see Diagram 1A.

After use, all culture glassware was washed in tissue culture detergent (7x - Flow Labs. Ltd.) and thoroughly rinsed free of detergent before being reassembled.

11:2 Double chamber vessels

These vessels were custom-made. The design was essentially that of single vessels, except that there were two inner chambers, one inside the other. The smaller of the two vessels had an internal diameter of 8 mm and the larger chamber was 21 mm in diameter. The lower end of the smaller inner vessel was covered with nucleopore membrane (Acropor: pore size 0.20 μ - Gelman Instrument Company, Ann Arbor, U.S.A.) held in place with washed rubber bands and the larger inner vessel was sealed at its lower end with the dialysis membrane used for the single chamber vessels. All membranes were boiled twice in distilled water before use. For further details of the culture vessels see diagram 1B.

12. 2-Mercaptoethanol (2-ME) was supplied by Koch-Light Labs. Ltd., Colnbrook, Bucks., England. Various dilutions were made in culture medium.

13. White cell counting fluid - Turk's blood counting fluid for white cells was prepared thus:-

Gentian violet 1% aqueous	1 ml
Glacial acetic acid	1 ml
Water to	100 ml

1. Preparation of cell suspensions

1:1 Spleen cell suspensions

Mice were killed by cervical dislocation and, if the spleens were being used for tissue culture, dipped into Hibitane. Spleens were removed aseptically and placed in a Petri dish containing medium. The spleens remained in the dish at room temperature until (1) enough spleens had been obtained to perform the experiment and (2) any other cells such as peritoneal cells had been harvested. Therefore, the lag time between removal of the spleens and further processing varied from 5 minutes to 1 hour. This did not seem to affect the viability of the cell suspensions produced. To produce a single cell suspension, the spleens were placed in fine wire mesh sieves which were fitted onto a centrifuge tube or a sterile Universal container, chopped into small pieces with sterile scissors and pressed with the plunger of a sterile 1 ml syringe. Freed cells were frequently washed through the sieve with medium. The suspension was left to stand for about 1 minute to allow any clumps to settle, the supernatant was decanted off and spun for 5 minutes at 1000 rpm at $4^{o}C$. The cells were resuspended in 10 ml medium and 0.1 ml of the suspension was added to 0.9 ml of counting fluid and counted in a haemocytometer. The viability of the cell suspension was assessed using the fluorescent method (to be described later in this section). Spleen cells were generally added to single Marbrook vessels at a concentration of 20 x 10^{6} cells/culture vessel. Other cell concentrations will be discussed in the text as appropriate.

When preparing spleen cell suspensions for experimental groups, cells from at least 3 spleen cell donors were pooled.

1:2 Peritoneal cells

Mice were killed by cervical dislocation, dipped in Hibitane and pinned ventral surface uppermost on a cork board. The skin was removed from the ventral and lateral surfaces of the mouse and pinned out of the

way, thus revealing the abdominal wall. To remove the peritoneal cells, 2 ml sterile medium was injected into the peritoneal cavity along the mid-line of the mouse. The abdomen was gently massaged for 1-2 minutes and then the fluid was removed. The easiest way to do this was to draw the wall of the abdomen away from the intestines etc., with the syringe needle, so as to form a pocket into which the fluid may run and then draw the fluid from this pocket. It was possible to obtain 1.7-1.9 ml of the injected fluid this way. The peritoneal cells were kept on ice in sterile Universal containers until they were added to cultures.

1:3 Fractionation of spleen cells into adherent and non-adherent populations

Spleen cell suspensions, prepared as described in Section 1:1, at a concentration of 20 x 10^6 cells/ml and a total volume of 20-25 ml were inoculated into 300 ml medicine bottles. (With smaller bottles, a correspondingly lower volume was used.) These were then incubated for 30 minutes at 37°C in a humid atmosphere with 5% CO_2. At the end of the first 30 minutes, the bottles were turned over onto their other side and incubated for a further 30 minutes. When the hour's adherence time was up, the bottles were shaken and the non-adherent cells decanted off. In initial experiments involving cell fractionation these non-adherent cells were centrifuged for 5 minutes at 1000 rpm at 4°C. Then the cells were resuspended to a final concentration of 20 x 10^6 cells/ml i.e. the same concentration as initially went into the bottles. However, it was found that more clear-cut results were obtained if the non-adherent cell concentration was not readjusted. (This will be discussed at greater length in the results.) Therefore, when non-adherent cells were cultured, they were at concentrations between 11-14 x 10^6 cells/culture vessel. To obtain the adherent cells, the surface of the bottles was washed twice with fresh medium and the washings were discarded. Then 5 ml of medium was added and the adherent cells removed by scraping with a rubber policeman. The cells were spun at 1000 rpm for 5 minutes at 4°C and the cell

pellet resuspended in 2 ml medium. This was the adherent cell population. For every 20 x 10^6 spleen cells inoculated into the bottles, 1-1.5 x 10^6 adherent cells were obtained. Therefore, there was a cell loss of between 4.5-7.5 x 10^6 cells. The viability of adherent cells prepared this way was between 75-85%.

Initially, adherent cells were added to culture vessels at a concentration of 2-4 x 10^6 cells/culture but later it was found that 10^6 cells were just as efficient for reconstituting the immune response to thymus/macrophage dependent antigens e.g. SRBC.

1:4 Bone marrow cell suspensions

Mice were killed by cervical dislocation and the femurs, tibias and iliac crests removed. The bones were cleaned to remove all surplus muscle and the ends of the bones cut off. Medium was flushed through the cavity to remove the bone marrow. The cells were counted in a haemocytometer and their viability assessed by the fluorescence method (see below).

1:5 T-cell preparation

Nylon wool (Leuko-Pak Leukocyte Filter, Fenwal Labs., Illinois, U.S.A.) was soaked in physiological saline for 2 hours at 37°C and then rinsed three times in double distilled water over a period of two to three days and dried in a 37°C incubator.

A 60 ml disposable syringe barrel was packed with 6 g of washed nylon wool and the column rinsed with approximately 20 ml medium. The column was drained and put into a 37°C incubator, in a humid atmosphere of 5% CO_2, to equilibrate for 1 hour before loading the cells. A spleen cell suspension was prepared as described in 1:1 and loaded onto the column at 10 x 10^6 cells in 20 ml medium. The cells were washed into the nylon wool with 10 ml warm (37°C) medium. The column was incubated at 37°C and 5% CO_2 for 45 minutes. Keeping the column in the incubator, it was washed slowly with warm medium to remove the non-adherent cells. The flow rate was approximately 1 ml/minute. These cells contained 3-5% contaminating B cells (personal communication from Dr. P. Kilshaw).

2. Marbrook cultures

2:1 Setting up cultures

Spleen cell suspensions, prepared as previously described, were added to the inner chamber of single Marbrook vessels to give 20 x 10^6 cells in 1 ml of medium. The antigens used *in vitro* initially were SRBC or DNP-POL but in later experiments DNP-KLH and DNP-Ficoll were also used. SRBC were usually added to give 2 x 10^6 cells/culture and DNP-POL was present at a final concentration of 0.1 µg/culture, DNP-KLH was present at a final concentration of 1 µg/culture and DNP-Ficoll at a final concentration of 20 ng/culture. The use of other spleen and antigen concentrations will be discussed in the text as appropriate. The total fluid volume was never less than 1 ml in the insert but on occasions as much as 1.5 ml fluid was placed in the insert. The outer vessel was filled with approximately 10 ml medium. Care was taken to match the fluid level in the insert with that of the medium in the outer vessel. The cultures were incubated in a Salamander CO_2 Tissue Culture Incubator (supplied by Boro Laboratories Ltd., England) at 37°C in a humid atmosphere containing 5% CO_2. The usual length of time for culturing was 4 days but both longer and shorter periods have been used and these will be indicated in the text as appropriate.

Cultures performed in double Marbrook vessels involved different cell numbers; 30 x 10^6 cells were placed in the larger, outer chamber and 3 x 10^6 cells in the smaller inner chamber. The contents of the two chambers were separated by a cell-impermeable nucleopore membrane. The reservoir was filled with 30 to 40 ml of medium. Further details will be given in the text of the results as appropriate.

Cultures using spleen cells from mice other than CBA's were supplemented with 2-ME. The details of concentrations used will be indicated in the text as appropriate.

2:2 Harvesting Marbrook cultures

At the end of the culture period, the cells were harvested by

pipetting the medium in the insert up and down with a Pasteur pipette to resuspend the cells and then removing them. Furthermore, the insert was washed with medium from the outer vessel. The cells were spun at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in 1 ml of medium by agitating gently on a Vortex. The number of antibody producing cells (plaque forming cells, PFC) per culture was assayed by means of the Cunningham assay.

2:3 The Cunningham assay

This assay was first described by Cunningham and Szenberg (1968). Cunningham chambers were made by sticking two microscope slides together with double-sided sellotape. The chambers were filled with a mixture of harvested spleen cells, SRBC or DNP-SRBC, adsorbed guinea pig complement and medium. The mixture was prepared in the wells of micro-titre plates (Flow Labs. Ltd.) thus:-

For assaying direct (IgM) plaques:-

3 drops medium

1 drop complement

1 drop of either 20% SRBC or 10% DNP-SRBC

100 µl cells harvested from the cultures

A drop was that volume dispensed from a gauge 2 dropping needle when attached to a 2 ml disposable syringe. There were 80 drops to 1 ml; therefore 1 drop is 12.5 µl and each microtitre well contained a total volume of 162.5 µl.

The chambers were filled by capillary action and the edges sealed with melted Paraplast (Sherwood Medical Industries) kept at 55°C on a hot plate. The chambers held between 76-84 µl fluid so the average contents of a Cunningham chamber was taken to be 80 µl. Therefore the mixture in the microtitre wells was enough to fill 2 Cunningham chambers. For the purposes of calculation it has been assumed that every Cunningham chamber holds 50 µl of harvested cells.

The chambers were incubated at 37°C for 30-45 minutes and the

plaques counted with a magnification of x25 using dark field illumination on a Zeiss microscope. The counting was usually performed immediately after the incubation time was up. Plaques must definitely be counted within 1 hour of the end of the incubation period or they begin to fade and counting becomes difficult and hence the results less reliable.

The counts obtained were the numbers of PFC/50 µl cells. Therefore to obtain the number of PFC/culture i.e. 1 ml of cells, the number of PFC/chamber was multiplied by 20. Each culture was assayed separately and for any experimental group quadruplicate cultures were set up. The results were expressed as the mean number of PFC/culture ± standard error (n = 4).

3. Cell viability

This was assayed by the fluorescence method of Takasugi (1971). Five mg fluorescein diacetate (Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in 1 ml acetone and 0.05 ml of this solution was added to 10 ml saline and then 200 µg ethidium bromide (Sigma Chemical Co.) was added to the solution. Equal volumes of cells and the fluorescein diacetate: ethidium bromide mixture were mixed together and examined under U.V. illumination using a Zeiss microscope. Live cells fluoresced green by this method and dead cells red. A differential count of 200 cells was done to assess the viability of any cell population.

4. Thymectomy

Mice anaesthetised with Avertin were stretched ventral side uppermost on a cork board and their heads held back over a neck support by looping an elastic band over the front upper incisors. An incision about 0.5-1 cm long was made in the skin above the manubrium sterni. The tip of curved forceps was hooked under the manubrium and the bone cut through with small straight scissors. The two lobes of the thymus were removed by suction using a custom made pipette attached to a water vacuum pump. The

incision was closed using autoclips which were removed 4 or 5 days later.

5. X-irradiation

Mice were irradiated to the whole body from above in perspex chambers using a Siemens X-ray machine operating at 10 mA, 240 kV with a 1 mm Cu filter. The target distance was 50 cm and the dose rate in air was 43.1 R/min.

Cell suspensions were irradiated in 30 ml perspex tissue culture flasks (Nunclon Delta-Jobling Lab. Division, England) using the same X-ray machine operating with a target distance of 34 cm and a dose rate of 96.1 R/minute. Cells, before and after irradiation, were kept on ice until used in culture.

6. T-cell depleted mice

Mice thymectomised 2-3 weeks previously were whole body irradiated with 800 R and were reconstituted either the same day or 24 hours later. Bone marrow cells from syngeneic donors were used for the reconstitution. The thymectomised, irradiated mice were injected intravenously (via the lateral tail vein) with 5×10^6 cells in 0.2 ml medium.

7. T-cell reconstituted mice

Mice, which had previously been either thymectomized and irradiated (850 R: whole body) or just irradiated (800 R) were injected intravenously with nylon wool purified T cells. The mice were given 2×10^7 T cells in 0.2 ml medium.

8. Spleen cells reconstituted mice

Mice thymectomized and irradiated or just irradiated were injected intravenously with 2×10^7 cells in 0.2 ml medium.

9. Production of infected mice

9:1 Mycobacterium lepraemurium (Mlm)

Livers and spleens removed from Wright-Fleming mice, that had been infected for 16-20 weeks, were homogenised first in a Colworth Stomacher 80 (A.J. Seward and Co. Ltd., London) and secondly in a glass homogeniser. The preparation was centrifuged at 1500 rpm for 5 minutes to remove any clumps and the supernatant was centrifuged at 3000 rpm or faster for 5 minutes to obtain the bacteria. The bacilli were counted by the spot slide method of Hart and Rees (1960) and made up to the correct concentration in physiological saline. CBA mice used in these experiments were routinely injected intravenously with 10^9 Mycobacterium organisms in 0.2 ml saline.

9:2 BCG

Dried BCG vaccine was reconstituted with saline and a human dose was usually used. This was 0.1 ml containing 8×10^5 viable units per mouse. (N.B. There are approximately the same number of dead ones due to the lyophilization procedure.) In some experiments a BCG culture was obtained from Glaxo as a gift which contained 10^9 BCG/ml. This suspension was diluted with fresh culture medium to give mouse doses of 10^8, 10^7 and 10^6 in 0.1 ml. The mice were injected intravenously. The last dose i.e. 10^6 is equivalent to 0.1 ml of the human dose type from the ampoule.

9:3 Salmonella typhimurium

Bacteria were inoculated into Glucose broth (Southern Group Lab., Hither Green Hospital, London S.E.13) and a stationary overnight culture was diluted for injection in physiological saline and 10% glucose broth. The final dose required was injected in 0.1 ml. The number of bacteria present were calibrated by optical density and serially diluted to the appropriate dose. The dose was confirmed by viable counts done on MacConkey Agar plates.

9:4 Malaria

A tail blood smear from a mouse parasitised with P. yoelii was made

and the number of parasitised cells/10,000 red blood cells were counted. This gave the degree of parasitaemia. A drop of tail blood was added to saline containing 5 I.U. heparin/ml and the number of red blood cells/ml were counted in a haemocytometer. From these two counts it is possible to estimate the number of parasitised cells/ml of blood. Mice were routinely injected with 10^5 parasitised cells intravenously.

10. Statistics

The significance of difference between control and experimental group was calculated using the two-tailed Student/Welch's t-test (Cochran and Cox, 1957).

CHAPTER THREE - INVESTIGATION OF THE MARBROOK SYSTEM

Marbrook and others have laid down criteria (Marbrook, 1967; Diener and Armstrong, 1969) for the functioning of this system of *in vitro* antibody synthesis. However, as the system is seemingly controversial as regards the ease of obtaining good reproducible results it is useful to include here in the results a survey of the system and its parameters.

RESULTS

3:1 Effect of relative concentrations of spleen cells and SRBC for optimal antibody response in vitro

Cultures containing 10, 20 or 40 x 10^6 spleen cells were made with various numbers of SRBC (2, 5 or 10 x 10^6 SRBC/culture). All cultures were harvested on day 4 and the number of PFCs/culture was assayed by means of the Cunningham technique. At first sight, the results in Table 1 suggest that for the optimal *in vitro* antibody response one should use 40 x 10^6 spleen cells against 2 x 10^6 SRBC but, if the results are expressed in PFC/10^6 it can be seen that the optimal response was obtained by either 10 x 10^6 spleen cells against 5 x 10^6 SRBC or 20 x 10^6 spleen cells against 2 x 10^6 SRBC. The latter combination has been chosen as the standard spleen cell:antigen (SRBC) ratio for two reasons:-

1. With further *in vitro* experiments, this combination gave less variation in the results.

2. It concurs with the findings of Marbrook concerning the parameters of the system (Marbrook, 1967).

It is interesting to note that the antibody response to SRBC appears to have a definite peak and if too little or too great an antigenic dose was given a lowered response was observed.

3:2 Time course of the in vitro antibody response to SRBC

Cultures containing 20 x 10^6 spleen cells and 2 x 10^6 SRBC were harvested at daily intervals until day 7. Table 2 shows the response was maximal on day 4, though the difference between that and the response on day 3 was not significant. However, when performing a straight uncomplicated primary *in vitro* antibody response to SRBC the cultures were

Table 1. Effect of varying spleen cells and SRBC concentration on the in vitro antibody response

No. of cells/culture	No. of SRBC/culture	PFC/culture	PFC/10^6 cells
10×10^6	2×10^6	880 ± 80	88
	5×10^6	1280 ± 100	128
	10×10^6	520 ± 30	52
20×10^6	2×10^6	2210 ± 70	111
	5×10^6	940 ± 45	47
	10×10^6	1020 ± 85	51
40×10^6	2×10^6	2620 ± 45	66
	5×10^6	900 ± 145	23
	10×10^6	1260 ± 90	32

Table 2. Time course of the in vitro antibody response to SRBC

Day of assay	PFC/culture
1	140 ± 18
2	925 ± 33
3	1775 ± 175
4	1980 ± 100
5	720 ± 50
6	460 ± 40
7	125 ± 46

harvested on day 4. Experiments where cultures were harvested on other days will be noted in the text as appropriate.

3:3 The optimal concentration of DNP-POL for the in vitro antibody response

20×10^6 spleen cells were cultured with doses of DNP-POL ranging from 1 μg to 0.001 μg and were harvested on day 4. The results are shown in Table 3. Optimal antibody responses were obtained using either 0.1 μg or 1 μg DNP-POL. Routinely in all the following experiments 0.1 μg DNP-POL was used as the in vitro antigenic dose. The response to DNP-POL was always 60-70% that of the response to SRBC.

3:4 The optimal concentration of DNP-Ficoll for the in vitro antibody response

Cultures containing 20×10^6 spleen cells were set up using 10-fold dilutions of DNP-Ficoll ranging from 2 μg to 0.002 μg and were harvested on day 4. The results, in Table 4, show that the optimal concentration of DNP-Ficoll was 0.02 μg/1 ml of culture fluid and this dose was used in experiments involving DNP-Ficoll. DNP-Ficoll was only used when the antigenicity of DNP-POL became doubtful. As will be shown later, DNP-POL and DNP-Ficoll are both macrophage independent antigens.

3:5 The optimal concentration of DNP-KLH for the in vitro antibody response

Spleen cells from mice previously primed with KLH were cultured with various amounts of DNP-KLH for 4 days. The results are shown on Table 5. It can be seen that the optimal response to DNP-KLH was obtained using 1 μg of the antigen in culture. If the mice were not previously primed with KLH, the response to DNP-KLH was no more than 100 PFC/culture. The reason for priming the mice is that it increases the number of cells capable of responding to the carrier (i.e. helper T cells).

3:6 Comparison of the ability of spleen cells from various mouse strains to give an in vitro antibody response to SRBC

Routinely spleen cells from CBA female mice were used in all experi-

Table 3. Effect of concentration of DNP-POL in the in vitro antibody response

DNP-POL concentration per culture (μg)	PFC/culture
0.001	580 ± 80
0.01	900 ± 80
0.1	1440 ± 30
1	1440 ± 80

Table 4. Effect of concentration of DNP-Ficoll in the in vitro antibody response

DNP-Ficoll concentration per culture (μg)	PFC/culture
0.002	846 ± 105
0.02	1706 ± 139
0.2	336 ± 33
2	760 ± 99

Table 5. Effect of concentration of DNP-KLH in the in vitro antibody response

DNP-KLH concentration per culture (μg)	PFC/culture
10	1310 ± 116
1	1780 ± 102
0.1	796 ± 149

Table 6. Response of spleen cells from various mouse strains to SRBC in vitro

Mouse strain	PFC/culture	
	With SRBC	Without SRBC
CBA	2016 ± 147	54 ± 21
C57/Bl	40 ± 18	30 ± 12
C3H	100 ± 42	69 ± 31

Table 7. Effect of adding various concentrations of 2-Mercaptoethanol (2-ME), to spleen cells of different mouse strains, on their ability to respond in vitro to SRBC

Mouse strain	2-ME conc.*	PFC/culture
CBA	Nil	2194 ± 69
	5×10^{-5}M	2334 ± 84
	10^{-4}M	2299 ± 154
C57/Bl	Nil	61 ± 42
	5×10^{-5}M	1590 ± 48
	10^{-4}M	1096 ± 71
C3H	Nil	79 ± 21
	5×10^{-5}M	216 ± 25
	10^{-4}M	1416 ± 99

* The concentration of 2-ME is expressed as the molar concentration present in the medium.

ments but the responses of spleen cells from other mouse strains have been studied. The two strains particularly studied were C57/Bl and C3H female mice. 20×10^6 spleen cells from CBA, C57/Bl and C3H mice were cultured against 2×10^6 SRBC for 4 days. Table 6 shows that a very poor response was obtained using either C3H or C57/Bl spleen cells in comparison with CBA cells. Their response was in fact not significantly above the normal background response of these strains to SRBC.

In view of the fact that many people (Click et al., 1972; Pike, 1975) use 2-mercaptoethanol (2-ME) in in vitro cultures to obtain a response the experiment was repeated using various concentrations of 2-ME in culture. The results are shown in Table 7. 2-ME enhanced the in vitro antibody response of C3H and C57/Bl spleen cells though their responses were never as good as those of CBA spleen cells. The optimal concentration of 2-ME was 10^{-4}M for C3H spleen cells and 5×10^{-5}M for C57/Bl spleen cells. It was interesting to note that the addition of 2-ME to cultures of CBA spleen cells did not greatly enhance their response to SRBC.

3:7 The effect of fractionation and reconstitution of spleen cells on the in vitro antibody response

Spleen cell populations were fractionated as described in the methods by means of their adherence to glass. Initially the concentration of nonadherent cells was readjusted to 20×10^6 spleen cells/ml and 1 ml of this suspension added to cultures; but later experiments using 1 ml of nonadherent cells at the concentration present after they had been allowed to adhere for an hour (i.e. not readjusted for cell loss) was shown to give clearer differences in the antibody responses of nonadherent cells alone and nonadherent cells reconstituted with adherent cells. The nonadherent cells were supplemented with various numbers of either spleen adherent cells or peritoneal cells. Tables 8 and 9 show results from experiments early in the developement of this technique and later when the conditions were more fully evaluated. From these tables, it can

Table 8. The effect of fractionation and reconstitution of spleen cells on the in vitro antibody response to SRBC (Early results)

Cells in culture*				PFC/culture
Spleen			Peritoneal cells	Anti-SRBC
Unfractionated	Non-adherent	Adherent		
20×10^6	-	-	-	2046 ± 77
-	-	-	10^6	0
-	-	-	2×10^6	0
-	-	-	4×10^6	0
20×10^6	-	-	2×10^6	3186 ± 95
-	13×10^6	-	-	140 ± 44
-	13×10^6	-	2×10^6	776 ± 62
-	13×10^6	-	4×10^6	2396 ± 300
-	20×10^6	-	-	1030 ± 132
-	20×10^6	-	2×10^6	2046 ± 97
-	-	10^6	-	46 ± 29
-	-	4×10^6	-	54 ± 28
-	13×10^6	10^6	-	1780 ± 69
-	13×10^6	10^6	10^6	2376 ± 91
Background (20×10^6 with no SRBC)				60 ± 10

* Numbers in the columns indicate the number of cells of that type added to the cultures.

Table 9. The effect of fractionation and reconstitution of spleen cells on the in vitro antibody response to SRBC (Later results)

Cells in culture*				PFC/culture
Spleen			Peritoneal cells	Anti-SRBC
Unfractionated	Non-adherent	Adherent		
20×10^6	-	-	-	2218 ± 103
-	12×10^6	-	-	70 ± 17
-	20×10^6	-	-	840 ± 124
-	-	-	10^6	0
-	-	10^6	-	20 ± 8
-	12×10^6	10^6	-	1700 ± 42
-	12×10^6	-	10^6	1864 ± 204
-	20×10^6	10^6	-	1929 ± 129
-	20×10^6	-	10^6	2024 ± 43
20×10^6	-	-	10^6	2568 ± 143
Background				80 ± 62

* Numbers of the columns indicate the number of cells of that type added to the culture.

be seen that using nonadherent cells which had not been readjusted, the differences between whole spleen cell populations and fractionated cells was more marked. Reconstituting such nonadherent cells led to an increase in the numbers of PFC/culture but this restoration could never be complete when compared to the unfractionated population. No PFC response could be found in either the adherent cell population or the peritoneal cells when compared to the background responses. Nonadherent spleen cells which had been readjusted to give a cell concentration of 20 x 10^6 cells/ml could be reconstituted to the same level as unfractionated spleen cells with either adherent spleen cells or peritoneal cells. There appeared to be no difference between reconstitution using peritoneal cells and spleen adherent cells. When this technique was being developed the number of adherent/peritoneal cells added to cultures varied from 10^6 to 4 x 10^6 and the best results were obtained using the higher numbers of adherent cells but with time it was found that using only 10^6 adherent cells the results were just as good. By fractionation of spleen cells into nonadherent and adherent populations, it is possible to lower the response of nonadherent cells to SRBC to a value only marginally higher than that of the background response. This response can be 80-90% reconstituted by the addition of either adherent spleen cells or peritoneal cells. This bears out the findings of various groups (Mosier, 1967; Sjöberg *et al.*, 1972) that the response to SRBC requires macrophages (the adherent cell population).

Another interesting fact to emerge from these reconstitution experiments was that when peritoneal cells were added to unfractionated spleen cells an enhanced PFC response was shown. This was a consistent finding and seems to be in direct apposition to the fact that excess macrophages can depress an immune response (Parkhouse and Dutton, 1966). It could however be explained by the fact that peritoneal cells contain between 30-50% T cells (Raff and Owen, 1971) which may in fact be able to provide extra help in *in vitro* antibody response.

Table 10. The effect of fractionation and reconstitution of spleen cell populations on the in vitro antibody response to DNP-POL

Cells in culture*				PFC/culture
Spleen			Peritoneal cells	Anti-DNP
Unfractionated	Non-adherent	Adherent		
20×10^6	-	-	-	1430 ± 68
-	13×10^6	-	-	1343 ± 42
-	20×10^6	-	-	1486 ± 60
-	13×10^6	10^6	-	1386 ± 103
-	13×10^6	-	10^6	1324 ± 43
-	20×10^6	10^6	-	1489 ± 133
-	20×10^6	-	10^6	1401 ± 65
20×10^6	-	-	10^6	1399 ± 120

* Numbers in the columns indicate the number of cells of cells of that type added to the cultures.

3:8 The effect of fractionation and reconstitution of spleen cell populations on the in vitro antibody response to DNP-POL

Nonadherent cells, both readjusted to 20 x 10^6 cells in culture and unadjusted (13 x 10^6 cells/culture), were cultured both with and without adherent or peritoneal cells in the presence of DNP-POL. From Table 10 it can be seen that the response to DNP-POL was not significantly depressed when cell fractionation was carried out. Nonadherent cells alone responded just as well as whole spleen cell populations and the response was not enhanced even by the addition of 10^6 peritoneal cells to normal unfractionated spleen cells. This confirms that the response of spleen cells to DNP-POL is independent of the presence of macrophages (Diener et al., 1970).

3:9 The effect of fractionation and reconstitution of spleen cell populations on the in vitro antibody response to DNP-Ficoll

Table 11 shows the results of a similar experiment as in 3:8 only using DNP-Ficoll as the antigen. The same phenomenon was observed as with DNP-POL, namely that nonadherent cells responded as well as unfractionated spleen cells. Therefore, it was concluded that DNP-Ficoll does not need the presence of macrophages to elicit an antibody response. This is in agreement with other findings (Mosier et al., 1974).

3:10 Effect of in vitro X-irradiation of spleen cell populations on the in vitro response to SRBC

Spleen cell suspensions were prepared as for use in cultures at a concentration of 20 x 10^6 cells/ml and subjected to doses of X-irradiation ranging from 100 R to 2000 R as described in the methods. These cells were then cultured for 4 days. As can be seen from Table 12 the in vitro response was completely suppressed by either 1000 R or 2000 R and 500 R achieved a considerable suppression. On this basis, a further experiment was carried out to locate the radiosensitive population. A spleen cell suspension was fractionated into the adherent and nonadherent cell populations and these two populations were irradiated with 600 R. Unfraction-

Table 11. The effect of fractionation and reconstitution on spleen cell populations on the in vitro antibody response to DNP-Ficoll

Cells in culture*				PFC/culture
Spleen			Peritoneal cells	Anti-DNP
Unfractionated	Non-adherent	Adherent		
20×10^6	-	-	-	1804 ± 71
-	13×10^6	-	-	1699 ± 43
-	20×10^6	-	-	1790 ± 104
-	13×10^6	10^6	-	1675 ± 84
-	13×10^6	-	10^6	1690 ± 31
-	20×10^6	10^6	-	1841 ± 63
-	20×10^6	-	10^6	1800 ± 13
20×10^6	-	-	10^6	1774 ± 59

* Numbers in the columns indicate the number of cells of that type added to the cultures.

Table 12. The effect of various doses of X-rays on the in vitro anti-SRBC response

Unfractionated spleen cells	Dose of X-rays	PFC/culture Anti-SRBC
20×10^6	Nil	1992 ± 136
20×10^6	100R	1868 ± 126
20×10^6	500R	288 ± 100
20×10^6	1000R	0
20×10^6	2000R	0

Table 13. Effect of 600R X-irradiation on the ability of spleen cell populations to reconstitute the in vitro response to SRBC

Unfractionated spleen cells	Non-adherent	Adherent	PFC/culture Anti-SRBC
Normal	-	-	2280 ± 100
Irradiated	-	-	125 ± 66
-	Normal	-	225 ± 44
-	-	Normal	0
-	Normal	Normal	1776 ± 34
-	Irradiated	-	0
-	-	Irradiated	0
-	Irradiated	Irradiated	0
-	Irradiated	Normal	20 ± 16
-	Normal	Irradiated	1700 ± 268

ated cells were also subjected to 600 R X-irradiation. These populations were then recombined in various ways. The results are presented in Table 13. The PFC response of spleen cells irradiated with 600 R was reduced to background levels and if irradiated nonadherent spleen cells were reconstituted with normal adherent cells the response was still only at background level. However if normal nonadherent cells were reconstituted with irradiated adherent cells, the response was comparable to that of reconstituting normal nonadherent cells with normal adherent cells. This in fact confirmed what others have found concerning the radioresistance of macrophages (Roseman, 1969; Sjöberg et al., 1972).

DISCUSSION

This section was mainly concerned with defining the parameters of the Marbrook system and before a discussion of the results is attempted a summary of the findings will be listed thus:-

1. The optimal spleen cell number for the primary antibody response *in vitro* was 20 x 10^6 spleen cells.

2. The optimal antigenic doses were 2 x 10^6 SRBC, 0.1 µg DNP-POL, 0.02 µg DNP-Ficoll and 1 µg DNP-KLH.

3. The peak of the *in vitro* anti-SRBC response was day 4.

4. By fractionation of spleen cells on the basis of their adherence to glass, it was possible to demonstrate macrophage dependency of the *in vitro* antibody response to SRBC but not to DNP-POL or DNP-Ficoll.

5. By combination of X-irradiation and cell fractionation, it was possible to show that the *in vitro* response was abrogated by 500 R or more. This radiosensitivity lay in the nonadherent cell population, the adherent cell population being radioresistant.

6. Spleen cells from moune strains other than CBA could be made to respond *in vitro* by the addition of 2-ME to the cultures.

In vivo veritas, though a misquotation, is certainly a truism, but if it were not for the development of *in vitro* techniques, the cellular mechanisms of immunology would not be so clearly defined as they are today. However, when using an *in vitro* system the relevance of any findings to the *in vivo* situation must be clearly assessed. The first obvious point of similarity between the *in vivo* and the *in vitro* IgM antibody response to SRBC is that they both have a peak on day 4. Also, many *in vivo* phenomena can be reproduced *in vitro* and this will be discussed in greater detail in the following chapters. However, one example will be mentioned. The *in vivo* enhancement of the antibody response to SRBC by *C. parvum* pretreatment is well known (Warr and James, 1975). *C. parvum* pretreated spleen cells when cultured *in vitro* were found to give an enhanced response to SRBC. Therefore, the system seems capable of reproducing *in vivo* findings.

A point of difference with respect to the *in vivo* situation is that using a Marbrook system it is not possible to observe an IgG response that is any greater than the preceding IgM response *in vitro* (Feldmann *et al.*, 1974). This is true even using primed spleen cells. Pierce (1969), using the Mishell-Dutton system for studying the *in vitro* antibody response was able to obtain an IgG response *in vitro* that was above the level of the IgM response by priming mice 5 to 10 days before using their spleen cells in culture. Points to be raised are that they were using different antigens, Pierce using SRBC and Feldmann using DNP-KLH. However, even when SRBC are used in the Marbrook system, I failed to produce a significant IgG response *in vitro* in cells primed to SRBC in a manner similar to Pierce's (results not shown in this thesis). Also, the Cunningham method used by Feldmann to assay his PFC response causes a 30% suppression of IgG plaques, due to the addition of complement at the start of the incubation period (Dresser and Greaves, 1973). Thirdly, Mishell-Dutton cultures are performed in a much smaller volume than are Marbrook cultures and so any factor(s) needed to cause the switch from IgM to IgG production, if they are dialysable, may be diluted out in the larger volume in which Marbrook cultures are performed. With respect to this question, Feldmann *et al.*, (1975) have shown that by addition of immunoglobulin extracted from T cell lines (E14 and WEHI 22), it was possible to elevate the IgG response to both DNP-POL and DNP-KLH in Marbrook cultures using primed cells. This factor however is nondialysable due to its molecular size and so would remain in contact with the cells during the whole culture period.

From personal experience, the Marbrook culture system seems very sensitive to any change in environment any time during the culture period. Differences in the pH or temperature during the last 24 hours of culture were just as detrimental as those on the first day of culture. In two years of performing this technique, there has been a gradual improvement in the response of these cultures and a lessening of the variation between

quadruplicate cultures. This is probably due to increased efficiency of setting up the cultures and also the innovation of a good CO_2 incubator. It definitely seems that a better operator performance was important when carrying out cell fractionation so that the cell viability was maintained. Also, the same bottles were used for carrying out cell fractionation and it appeared that there was a certain time needed for glass surfaces to be conditioned before they support the growth of cells adequately. This conditioning is often done by treating the bottles chemically and then growing continuous cell lines in them (Rappaport, 1960).

The immunogenicity of the antigen is also important for obtaining good consistent results. DNP-POL, for instance, after 18 months use in culture ceased to produce any response above background levels in culture (results not shown) and at any dose tested in culture was no longer immunogenic. Again, using DNP-Ficoll, when this antigen was first assayed in culture, the optimal dose was 0.002 µg. On reassaying, one year later, the optimal dose for use in culture was 0.02 µg.

The evidence from cell fractionation experiments seems to bear out the findings of others as to the macrophage dependency of some antigens and the macrophage independency of others. SRBC were found to be macrophage dependent in agreement with Mosier (1967) and Sjöberg *et al.*, (1972). DNP-POL and DNP-Ficoll were found to be macrophage independent in agreement with the work of Diener *et al.* (1970) and Mosier *et al.* (1974) respectively. However, recent work by Lee *et al.* (1976) has questioned such conclusions. Using carbonyl iron to separate their cell populations and comparing the results to those obtained when adherence to glass was used as the criterion for cell separation, they found that there was a need for adherent cells in the responses to soluble SRBC antigen, DNP-Ficoll and POL *in vitro*. However, the dependence of these antigens on such an adherent cell was less than that of the response to intact SRBCs. They suggest in their results that this requirement for adherent cells was not due to poor cell viability after cell separation as the viability

of treated cells and the controls was similar. Nor was it due to a loss of immunocompetent cells as the numbers of theta and immunoglobulin bearing cells and antigen binding cells after separation procedures remained unaltered. Although altering the way of thinking about antibody production by the classical macrophage independent antigens, it still does indicate a difference in the degree to which macrophages are required. For the purposes of the results to be demonstrated in the following sections it would not appear to be too critical a finding as will become apparent.

During this series of experiments the macrophage requirement of DNP-KLH was not assessed. The macrophage dependence of this antigen is open to dispute (Katz and Unanue, 1973) and therefore it would have been profitable to test its macrophage requirements for myself. If it eventually was the case that the response to DNP-KLH did not require macrophages but did require T cells, it would be very useful as a tool for further separating the complex web of cell interactions. Though the findings of Lee et al. (1976) make it very difficult to envisage an antigen which while requiring T cells did not require macrophages.

A discrepancy with the work of others (Parkhouse and Dutton, 1966) is that I have never been able to demonstrate a suppression of the immune response by the addition of excess macrophages. The critical figure for observing macrophage suppression is said to be when they are added in excess of 5% of the total cell number. A number of macrophages equivalent to this percentage has never been added to unfractionated spleen cells but 4×10^6 peritoneal cells have been added to 13×10^6 nonadherent spleen cells and this has only ever been shown to reconstitute the in vitro immune response. Also, when 10^6 peritoneal cells were added to 20×10^6 unfractionated spleen cells, on one occasion a slight enhancement of the in vitro immune response was observed. It is of course possible to postulate that the depression observed in other systems was due to the production of a dialysable factor which would not be effective in the

Marbrook system. Also, the addition of peritoneal cells to the cultures means that T cells are also being added, as mouse peritoneal cells contain 30-50% T cells (Raff and Owen, 1971). Thus the addition of peritoneal cells may mean that extra help in the form of T cells is being added to the system and hence the enhancement noted in my experiments.

The experiments on the effect of irradiating different cell populations seems to bear out the findings of others that macrophages are the radioresistant cell population and that lymphocytes are the radiosensitive cell population (Roseman, 1969; Sjöberg et al., 1972). Such a criterion is useful for delineating more exactly which population is effecting a certain function. These results also confirm that separation of cell populations by their adherence to glass does actually describe two distinct cell populations. This is supported by the finding that the adherent cells never produced antibody when cultured in vitro for 4 days. Antibody production was not observed by peritoneal cells in culture which is at variance with results reported by Bussard and his co-workers (Lowy et al., 1975).

The addition of 2-ME has been shown to potentiate the antibody response to SRBC of mouse strains other than CBA. The mechanism of this potentiation was not studied in the present work. Chen and Hirsch (1972a) found that it was possible to completely replace the need for adherent cells in in vitro anti-SRBC responses by the addition of 2-ME to nonadherent cell populations. They suggested that the primary action of 2-ME on the nonadherent cells was in promoting their viability and reactivity (Chen and Hirsch, 1972b). There are reports that C57/Bl spleen cells produce cytotoxic products in cell culture (Kramer and Granger, 1972). Therefore, it is possible that 2-ME acts in such a way as to abrogate the effects of such cytotoxic agents by supporting the viability and reactivity of the lymphoid cells. It is possible that the same mechanism can be postulated for 2-ME's potentiation of the response of C3H spleen cells in culture.

This therefore is a brief analysis of the parameters of the Marbrook system that will be used throughout this thesis to study the effect of nonspecific macrophage activation by the adjuvant C. parvum, infections such as M. lepraemurium, S. typhimurium, BCG and malaria and carrageenan, which is reported to be cytotoxic for macrophages, on the in vitro antibody response to both T dependent and independent antigens.

CHAPTER FOUR - EFFECTS OF *C. PARVUM* ON THE *IN VITRO* ANTIBODY RESPONSE

Immunologic interest in the gram-positive anaerobe C. parvum was aroused following the demonstration of its remarkable stimulatory effect on the mononuclear phagocyte system (Halpern et al., 1964). They found that a single intravenous injection of C. parvum when given as a killed vaccine caused a marked hepato- and splenomegaly of approximately 3 weeks duration. The phagocytic ability of these C. parvum treated animals, as measured by carbon clearance, was also markedly enhanced. There have also been reports that pretreatment with C. parvum leads to an increased nonspecific resistance to various infections. The first report was the work of Nussenzweig (1967) who showed that 1 to 3 weeks following systemic injection of C. parvum, mice showed an increased resistance to challenge with the sporozoites of Plasmodium berghei. Enhanced resistance to various bacterial (Adlam et al., 1972; Collins and Scott, 1974) and viral infections (Cerutti, 1975) following C. parvum pretreatment have been reported. Frost and Lance (1973) have also reported that C. parvum is one of the strongest inducers of lymphocyte trapping.

There is a plethora of information regarding the protection against tumours afforded by C. parvum and the subject of C. parvum as an immunotherapeutic anticancer agent has been admirably reviewed by Scott (1974b). Therefore only a brief mention of this field of study will be made. The first report of C. parvum's systemic antitumour efficacy was in 1966 (Woodruff and Boak). They found that C. parvum delayed the appearance of a subcutaneously injected spontaneous mammary carcinoma. However, once the tumour became palpable the subsequent growth rate was unaffected. Later studies showed that a single intraperitoneal injection of C. parvum 3 days after tumour establishment inhibited the growth of the same tumour and two other unrelated ones (Woodruff et al., 1972). C. parvum has also been injected directly into the tumour lesion causing a rapid and lasting regression of both the primary tumour and its metastases (Likhite and Halpern, 1974).

As well as this nonspecific enhancement, there are now reports of the depressive effects of systemic C. parvum injection with regard to various T cell mediated immune phenomena. Reports of impaired delayed hypersensitivity (Asherson and Allwood, 1971; Allwood and Asherson, 1971, 1972; Scott, 1974), PHA, mixed lymphocyte and graft versus host responses (Scott, 1972) and homograft reactions (Castro, 1974) following C. parvum pretreatment have all been noted.

That C. parvum is an adjuvant in the classical sense of giving rise to an enhanced antibody response has been demonstrated many times. Neveu et al. (1964) found that heat killed C. parvum added to Freund's incomplete adjuvant increased the immunological responsiveness of guinea pigs to picrylated proteins in terms of an increased antibody production and the induction of a delayed type hypersensitivity to these proteins. Biozzi and his co-workers (Biozzi et al., 1968) found that C. parvum pretreatment increased the primary response to SRBC in terms of both the rosette forming cells and the haemagglutin titres. Pretreatment of rabbits with C. parvum caused an increased response to bovine serum albumin. This augmentation was reflected in the quantity of antibody and the more rapid evolution of the relative binding affinity of the antibodies (Pinckard et al., 1967). The response to T independent antigens has also been shown to be enhanced (Howard et al., 1973a). They found that C. parvum pretreatment exerted a strong adjuvant effect on the response to pneumococcal polysaccharide (S III). The adjuvant effect of C. parvum however was dependent on the dose of S III. Del Guercio (1972) also reported that C. parvum was capable of stimulating the B cell responses to DNP-Levan, another thymus independent antigen which appears to need or involve both DNP and Levan specific B cells (del Guercio and Leuchars, 1972). Howard and his co-workers also studied the effect of C. parvum pretreatment on the response to SRBC and rat red cells in mice in terms of the effect on the haemagglutin and PFC responses (Howard et al., 1973b). They found that both the 19S and 7S components of the

response were increased by C. parvum treatment, but that the enhancement of the 19S response persisted longer and could be elicited by normally subimmunogenic doses of antigen.

The hypothesis that macrophages are involved in any of the effects of C. parvum has emerged over a period of time. The early observations that C. parvum pretreatment caused an enhanced phagocytic ability (Halpern et al., 1964) have been added to from all angles both in vivo and in vitro. Macrophages from C. parvum treated animals behave like those activated by other means in that they show accelerated adherence to glass, vacuolation and increased lysosomal enzyme activity (Olivotto and Bomford, 1974). Scott (1972b) showed that the depression of the PHA response of cells from C. parvum pretreated animals was due to macrophages and that normal responses could be obtained if the adherent cells were removed from the spleen cells. Frost and Lance (1973) have evidence suggesting that the enhancement of lymphocyte trapping observed in C. parvum animals is macrophage mediated. Howard et al. (1973b) postulate that it is the activated macrophage that mediates all the responses they observe. This is based on somewhat circumstantial evidence such as the fact that C. parvum is phagocytosed within a very short time of systemic injection and that the lag period before the onset of a demonstrable immunopotentiating effect coincides with the emergence of activated macrophages.

There was at the time this work was started no direct evidence that macrophages from C. parvum treated animals were capable of stimulating the response to SRBC and so with this aim in mind this work was started. During the course of it, Wiener (1975) produced evidence to the effect that macrophages from C. parvum animals were better at reconstituting the response of normal nonadherent cells to SRBC. However, the present study is a more complete analysis of the situation.

RESULTS

To study the adjuvant effect of C. parvum in vitro three approaches were used:-

1. Mice were pretreated with C. parvum and the spleen cells of these animals were used in Marbrook cultures to see the effect of such pretreatment on the in vitro antibody response.

2. C. parvum was added to untreated mouse spleen cells in vitro to elicit a completely in vitro system for studying the mechanism of C. parvum enhancement of the immune response.

3. The use of Marbrook double chambers.

4:1 The effect of pretreating mice with different doses of C. parvum on the in vitro antibody response to SRBC

Mice were injected intravenously with various doses of C. parvum 5 days prior to the removal of their spleens for use in culture. The doses given ranged from 1.40 mg to 0.14 mg dry weight of organisms. The results are shown in Table 14. It can be seen that all doses of C. parvum enhanced the in vitro antibody response and that this varied from 2 to 3.5 times. In view of the experience of Warr (1973) that higher doses of C. parvum were toxic and the fact that in the present experiment only a relatively small increase of the in vitro response with increasing doses was found, the dose of 0.35 mg was selected for further experiments.

4:2 Effect of time of administration of C. parvum on the antibody response of spleen cells in vitro

Mice were injected with 0.35 mg C. parvum at various times ranging from 2 hours to 21 days before taking the spleens for use in culture. As Table 15 shows the experiment was performed in two parts: one part for the intervals of 0 to 5 days and the second part of the experiment for 1, 2 and 3 week intervals. As can be seen even when C. parvum was given 1 day before, there was a slight enhancement in the antibody response that was significant at the 5% level. Maximal enhancement appeared to be between 5 and 7 days. However, the effect was present even at 21 days

Table 14. The effect of dose of C. parvum administered to mice on the antibody response of spleen cells in vitro to SRBC

Dose of C. parvum (mg)	PFC/culture Anti-SRBC
0	2010 ± 107
0.14	4436 ± 107
0.175	6550 ± 153
0.35	6400 ± 74
0.70	7060 ± 134
1.40	7200 ± 423

Mice injected 5 days before their spleens were removed.

Table 15. The effect of time of administration of C. parvum on the antibody response of spleen cells in vitro to SRBC

Days after C. parvum	PFC/culture Anti-SRBC	
	Exp. 1	Exp. 2
Nil	2010 ± 107	2064 ± 128
1/12*	2596 ± 140[a]	
1	2680 ± 53	
3	3786 ± 120	
5	5736 ± 174	
7		6044 ± 95
14		4828 ± 115
21		3712 ± 100

Mice were injected with 0.35 mg C. parvum intravenously.

* C. parvum injected 2 hours before taking spleen cells for use in culture.

a = P is < 0.05

after pretreatment of the animals, though at a lower level. The results are also shown as a graph, Fig. 1.

On the basis of these results, it was decided that routine procedure for the pretreatment of mice would be 0.35 mg dry weight of C. parvum organisms given 5 days before the spleen cells were taken for use in culture.

4:3 Effect of pretreatment with C. parvum on the in vitro antibody response to DNP-POL using various concentrations of DNP-POL

Spleen cells, from mice both untreated and treated 5 days previously with C. parvum, were cultured with various concentrations of DNP-POL to see if any enhancement such as obtained with SRBC could be found. The results are shown in Table 16. They indicate that pretreatment with C. parvum did not affect the response of spleen cells to DNP-POL. In all cases, except one, the response of pretreated cells was similar to or lower than the response of normal cells. However, with an antigenic dose of 0.01 µg DNP-POL, the response of C. parvum pretreated cells was almost twice that of untreated cells. This situation is very difficult to analyse because the normal cells were not responding optimally and so the apparent enhancement could be due to many factors. However the general conclusion is that C. parvum did not enhance the in vitro response to DNP-POL.

4:4 The effect of C. parvum pretreatment on the in vitro antibody response to DNP-KLH

Spleen cells, from mice both untreated and treated 5 days previously with C. parvum, were cultured with various doses of DNP-KLH to see if an enhancement such as obtained with SRBC could be found. Spleen cells from mice previously primed to KLH were also cultured. From Table 17, it can be seen that normal spleen cells responded poorly to DNP-KLH and that to obtain a good response the mice had to have been previously primed with KLH. However, when spleen cells from C. parvum treated animals were used the response was enhanced whether as compared to normal unprimed cells or

Table 16. The effect of pretreatment with C. parvum on the in vitro antibody response to DNP-POL using various concentrations of DNP-POL

Spleen cell source	DNP-POL (µg/culture)	PFC/culture Anti-DNP
Normal	0.01	450 ± 24
"	0.1	1516 ± 70
"	1.0	1126 ± 61
"	10.0	506 ± 49
C. parvum	0.01	880 ± 91
"	0.1	1546 ± 109
"	1.0	520 ± 50
"	10.0	566 ± 73

Fig. 1. Effect of time of administration of C. parvum on the antibody response of spleen cells in vitro

Open circles represent the PFC response of cells from C. parvum treatec animals ± S.E.

Cross-hatching represents the PFC response of cells from normal animals ± 95% confidence limits.

Fig. 2. Comparison of the time course of the in vitro responses of untreated and C. parvum pretreated cells to SRBC.

Upper line represents the PFC response of cells from C. parvum treated animals ± S.E.

Lower line represents the PFC response of cells from normal animals ± S.E.

FIG.1

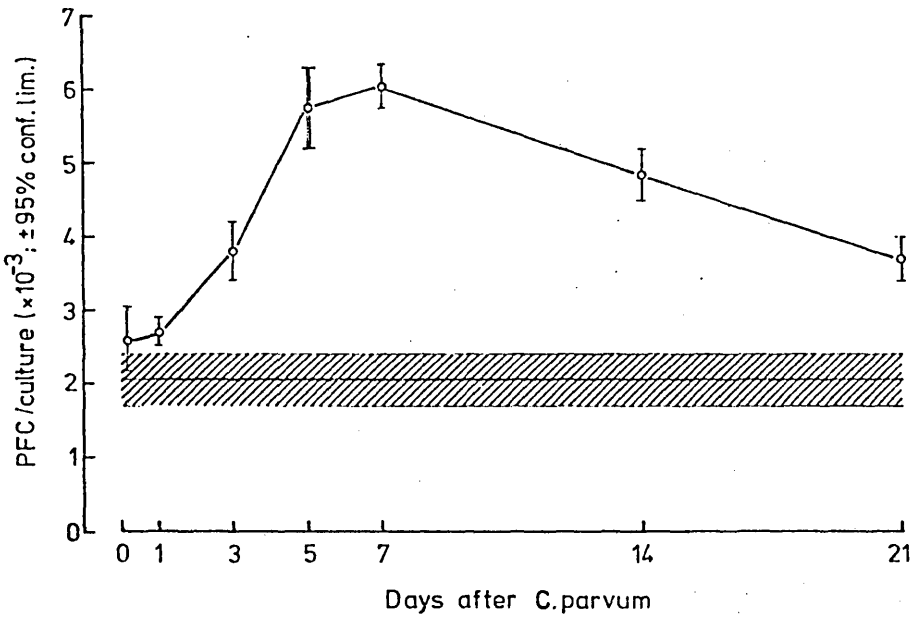

PFC/culture (×10⁻³; ±95% conf. lim.)
0
1
2
3
4
5
6
7
0 1 3 5 7 14 21
Days after C.parvum

FIG.2

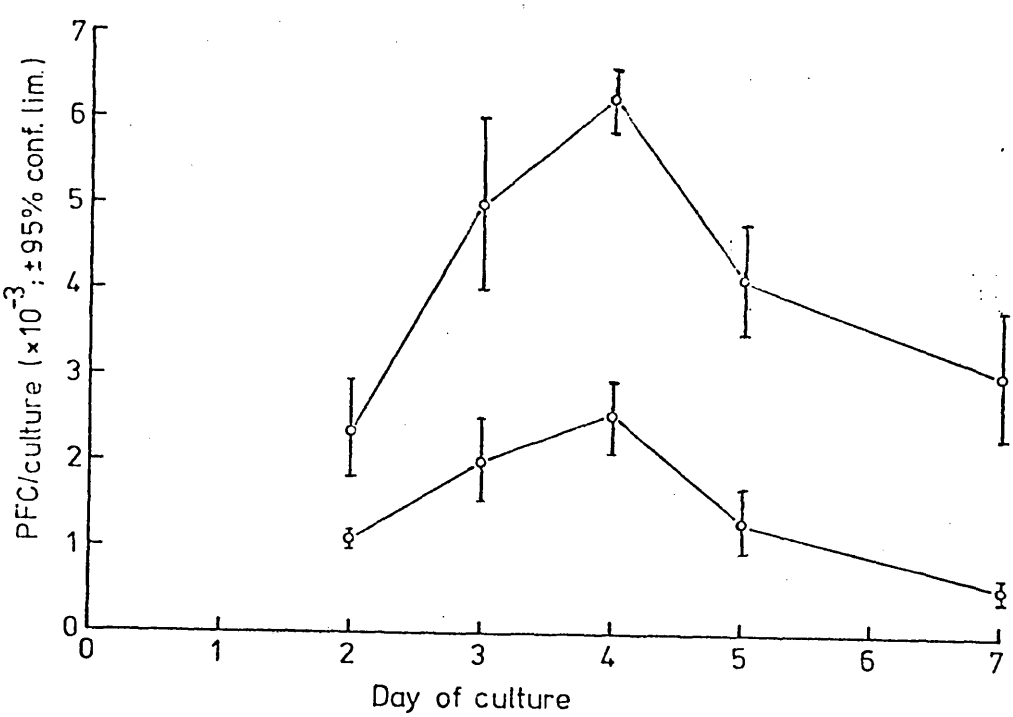

PFC/culture (×10⁻³; ±95% conf. lim.)
0
1
2
3
4
5
6
7
0 1 2 3 4 5 6 7
Day of culture

Table 17. The effect of pretreatment with C. parvum on the in vitro response to DNP-KLH

Source of spleen cells	Dose of DNP-KLH (µg/culture)	PFC/culture
Normal	0.1	336 ± 98
	1.0	162 ± 29
	10.0	270 ± 31
C. parvum	0.1	2506 ± 165
	1.0	3161 ± 349
	10.0	2212 ± 173
KLH primed	0.1	1149 ± 123
	1.0	2131 ± 104
	10.0	1800 ± 163

KLH primed indicates spleen cells primed to KLH as described in the methods section.

in comparison to primed spleen cells. This would indicate that C. parvum pretreatment also effects T cell as well as the macrophage function.

4:5 Comparison of the time course of the in vitro responses of untreated and C. parvum pretreated spleen cells to SRBC

When the enhancing effect of C. parvum pretreatment had been demonstrated in vitro the possibility that this pretreatment might alter the kinetics of the in vitro response to SRBC was studied. Therefore, untreated and pretreated spleen cells were cultured in the presence of SRBC for 2, 3, 4, 5 and 7 days. The results are shown in Table 18. These results are also represented in graphical form (Fig. 2). It can be seen that the pattern of the response of treated and untreated cells was the same. However even on day 2 the response of C. parvum pretreated cells was twice that of untreated cells. This enhanced response continued throughout the period under study though the peak of the response was on the same day in both the normal and treated spleen cells.

Another point to be noted was that C. parvum pretreatment did not cause any difference in the background responses of normal and pretreated cells with respect to SRBC (Table 18). This indicates that there was no cross reactivity between SRBC and C. parvum so that any enhancement observed was not due to pretreatment with C. parvum being the equivalent of priming the animals with SRBC.

4:6 Cellular basis of the enhancement induced by C. parvum

The findings that pretreatment with C. parvum enhanced the response to SRBC, but not to DNP-POL, suggested that this effect could be mediated by T cells and/or macrophages, since the response to the latter antigen is independent of both these cell types. In view of the well documented effects of C. parvum on the proliferation and phagocytic activity of macrophages in vivo these cells appeared to be the primary candidates for the enhanced response to SRBC.

To investigate this hypothesis, spleen cells from both untreated and C. parvum pretreated animals were fractionated into the adherent and

Table 18. Comparison of the time course of the in vitro anti-SRBC response in spleen cells from normal and C. parvum pretreated mice

Day of assay	PFC/culture of spleen cells from donors	
	Normal	C. parvum
2	1090 ± 27	2370 ± 178
3	2030 ± 148	4996 ± 32?
4	2530 ± 130	6226 ± 125
5	1310 ± 116	4166 ± 205
7	550 ± 55	3046 ± 236
Background	90 ± 34	100 ± 18

Background assayed by culturing both normal and C. parvum treated cells for 4 days in the absence of SRBC and then assaying for the number of PFC.

Table 19. Effect of pretreatment of mice with C. parvum on the antibody response of spleen cells (fractionated and recombined) in vitro

Spleen cells in culture			PFC/culture		
			Anti-SRBC response		Anti-DNP response
Unfractionated	Nonadherent	Adherent	Exp. 1	Exp. 2	Exp. 1
Normal	-	-	2050 ± 71	2190 ± 110	1446 ± 64
-	-	Normal	0	0	0
-	Normal	-	210 ± 13	140 ± 22	1236 ± 81
-	Normal	Normal	1861 ± 62	1676 ± 54	1200 ± 90
C. parvum	-	-	7200 ± 423	6780 ± 100	1450 ± 68
-	-	C. parvum	0	0	0
-	C. parvum	-	290 ± 55	110 ± 24	1486 ± 62
-	C. parvum	C. parvum	6443 ± 99	5856 ± 210	1566 ± 76
-	Normal	C. parvum	3310 ± 273	5536 ± 70	1616 ± 87
-	C. parvum	Normal	1816 ± 59	1820 ± 17	1536 ± 43

Cell types and numbers used in culture as follows:
Unfractionated = 20 x 10^6 spleen cells/culture
Nonadherent = 13-14 x 10^6 spleen cells/culture
Adherent = 2 x 10^6 cells/culture
Normal - spleen cells from untreated animals.
C. parvum - spleen cells from animals pretreated 5 days previously with 0.35 mg dry weight C. parvum organisms.

nonadherent cell populations and then recombined in various ways. The results are shown in Table 19. The first part of the table shows the situation previously demonstrated in Chapter Three of this thesis, i.e. fractionation of normal spleen cells caused the unresponsiveness of the nonadherent cell population to SRBC but this could be reconstituted by the addition of normal adherent cells. However, such procedures had no effect on the response to DNP-POL; both unfractionated and nonadherent spleen cells responded equally well to the antigen. No response to either antigen was found using the adherent cell population. The second part of the table shows the same procedures carried out on C. parvum pretreated spleen cells. Here it can again be seen that C. parvum pretreatment caused an enhancement of the response to SRBC but not to DNP-POL. C. parvum nonadherent cells were not significantly different in their response to either antigen when compared to untreated nonadherent spleen cells. The response to SRBC could be restored with the same degree of enhancement by recombining C. parvum nonadherent and adherent cells.

When normal nonadherent cells were reconstituted with adherent cells from C. parvum treated mice, the response was enhanced. If the reverse reconstitution was performed, C. parvum nonadherent cells reconstituted by normal adherent cells, the anti-SRBC response was normal, no enhancement could be observed. Neither of these procedures affected the response to DNP-POL. Further evidence for macrophages being involved in this enhancement was obtained when this experiment was repeated using cell populations subjected to 600 R X-irradiation. As shown earlier in the results of Chapter Three, there are cells in the adherent population which are radioresistant and are capable of restoring the PFC response to SRBC of unirradiated nonadherent cells. The nonadherent cells were radiosensitive and their ability to respond to SRBC was completely abolished by 600 R. The results of this experiment are shown in Table 20. The abrogation of the antibody response by 600 R was clearly demonstrated and it can also be seen that pretreatment with C. parvum did not alter the

Table 20. Effect of X-irradiation on the ability of spleen cell populations from C. parvum treated and untreated mice to reconstitute a functional antibody response in vitro

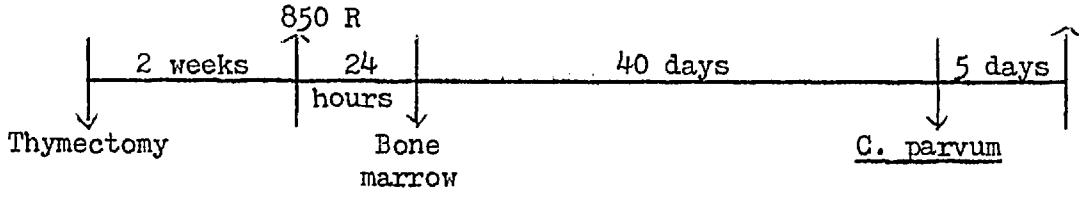
Spleen cells in culture			PFC/culture	
Unfractionated	Nonadherent	Adherent	Anti-SRBC	Anti-DNP
Normal	-	-	2280 ± 50	1446 ± 64
C. parvum	-	-	6780 ± 100	1450 ± 68
Normal-X	-	-	125 ± 33	-
C. parvum-X	-	-	300 ± 29	-
-	C. parvum-X	C. parvum	140 ± 20	0
-	C. parvum	C. parvum-X	5520 ± 70	1656 ± 76
-	C. parvum	Normal-X	1726 ± 35	1566 ± 76
-	Normal-X	C. parvum	40 ± 18	0
-	C. parvum-X	Normal	106 ± 30	0
-	Normal	C. parvum-X	5610 ± 138	1600 ± 110

Experimental groups designated thus:
Normal = untreated animals
C. parvum = animals given C. parvum 5 days previously
X = cells subjected to 60CR X-irradiation prior to use in culture
Cell numbers used in culture were the same as in Table 19.

relative radiosensitivities of adherent and nonadherent cell populations. C. parvum nonadherent cells were radiosensitive and in the presence of unirradiated adherent C. parvum cells did not give a response to either SRBC or DNP-POL. However, irradiated C. parvum adherent cells were capable of reconstituting unirradiated C. parvum nonadherent cells and giving an enhanced antibody response. Irradiated C. parvum adherent cells were also able to reconstitute and enhance the response of normal unirradiated nonadherent cells. These two experiments taken together indicate that macrophages from C. parvum treated animals were capable of producing an enhancement of the antibody response when recombined with either normal or C. parvum nonadherent cells. C. parvum nonadherent cells (lymphocytes) on the other hand did not seem to affect the performance of normal adherent cells.

4:7 T cell dependence of macrophage activation of cells from C. parvum pretreated animals in vitro

In view of reports that in other systems the activation of macrophages was T cell dependent (Mackaness, 1971), the possibility that this was also true in the case of C. parvum activation was investigated. Mice were thymectomized and 2 weeks later were whole body irradiated with 850 R and 24 hours later they were reconstituted with 5×10^6 bone marrow cells. These mice were then left for at least 40 days before being used in experiments. Animals were pretreated with C. parvum 5 days prior to the the cells being taken for use in cultures.



Protocol for pretreatment of animals

Table 21 shows the results of two such experiments. It can be seen that cells from thymectomized, X-irradiated, bone marrow reconstituted

Table 21. Lack of enhancement of the antibody response in vitro by macrophages from T cell depleted mice

Cells in culture				PFC/culture		
Spleen			Peritoneal cells	Anti-SRBC		Anti-DNP
Unfractionated	Nonadherent	Adherent		Exp. 1	Exp. 2	
Normal				2190 ± 89	2200 ± 116	1400 ± 99
C. parvum				6476 ± 320	4690 ± 273	1340 ± 83
TXB				560 ± 41		1396 ± 98
TXB-*C. parvum*				666 ± 107		1336 ± 59
	Normal	Normal		1810 ± 27	1650 ± 101	1520 ± 57
	Normal	*C. parvum*		5396 ± 208	3810 ± 166	1476 ± 106
	Normal	TXB		1600 ± 34	1710 ± 81	1365 ± 55
	Normal	TXB -*C. parvum*		1610 ± 58	1666 ± 81	1370 ± 89
	Normal	XB			1760 ± 109	
	Normal	XB-*C. parvum*			2970 ± 170	
	Normal		Normal	2500 ± 60	1746 ± 171	1446 ± 66
	Normal		*C. parvum*	5630 ± 128	3370 ± 170	1280 ± 105
	Normal		TXB	1270 ± 72	1826 ± 137	1420 ± 77
	Normal		TXB-*C. parvum*	1460 ± 135	1866 ± 43	1546 ± 77
	Normal		XB		1740 ± 73	
	Normal		XB-*C. parvum*		3026 ± 172	

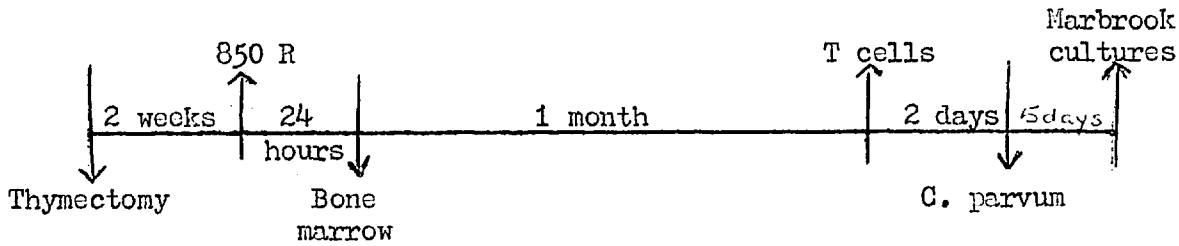
(TXB) donors gave a response only 25% that of normal cells in culture. However, cells from TXB donors responded as normal to DNP-POL, an antigen which is both macrophage and T cell independent. Spleen cells from the donors which had been pretreated in different ways were fractionated and the adherent cells of all these types were reconstituted with normal non-adherent cells. It can be seen that adherent cells from TXB animals were able to reconstitute normal nonadherent cells to give an almost normal response, as expected. However when adherent cells from TXB mice treated with C. parvum (TXB-C. parvum) were reconstituted with normal non-adherent cells they did not give a response any greater than untreated TXB adherent cells. This indicated that macrophages could not be activated in the absence of T cells. Macrophages from sham-thymectomized, X-irradiated, bone marrow reconstituted C. parvum treated (XB-C. parvum) mice though showing some enhancing activity were not as good as those from unirradiated C. parvum treated mice.

The same pattern of response was found when using peritoneal cells to reconstitute normal nonadherent cells. Throughout all these manipulations the response to DNP-POL remained unaltered.

These experiments were the first to show that for the activation of macrophages by C. parvum, it is necessary to have T cells present.

4:8 Recovery of the ability of macrophages in T cell depleted mice reconstituted with T lymphocytes to enhance the in vitro antibody response

When it was found that macrophages from animals lacking T cells could not be activated on pretreatment with C. parvum, it was decided to see if it was possible to restore the activation of the macrophages of these mice by giving them purified T cells. Mice were thymectomized and two weeks later irradiated with 850 R whole body X-irradiation and 24 hours later reconstituted with 5×10^6 bone marrow cells. After one month some of these animals were given 2×10^7 T cells intravenously. Two days later the animals were pretreated with C. parvum.



Time scale of pretreatment of animals

The results of this experiment are shown in Table 22. When unfractionated cells from the various groups of animals were cultured in vitro it was again found that spleen cells from both thymectomized and C. parvum pretreated thymectomized mice failed to give a normal response to SRBC due to the lack of T cells. When cells from animals which had been thymectomized and T cell reconstituted were cultured they gave an almost normal response and pretreatment of such cell donors with C. parvum led to an enhanced antibody response in vitro.

The next stage of this experiment was to fractionate the adherent and nonadherent cells of each cell donor type. Adherent spleen cells and peritoneal cells from all donor types were reconstituted in vitro with normal nonadherent cells. From the table it can be seen that it was possible to restore the enhancing ability of C. parvum treated adherent cells by prior injection of T cells. Only adherent cells or peritoneal cells from thymectomized, T cell reconstituted, C. parvum treated mice were able to give an enhancement similar to that effected by macrophages from animals only pretreated with C. parvum.

The final section of Table 22 shows clearly that when either unfractionated or nonadherent spleen cells from thymectomized mice were reconstituted with normal peritoneal cells there was an increased response to SRBC indicating T cell activity. This supports the suggestions of others that peritoneal cells contain T cells. One anomaly however did appear. When normal adherent spleen cells were used to reconstitute either unfractionated or nonadherent cells from thymectomized mice the response to SRBC was increased.

Table 22. Recovery of the ability of macrophages from T-cell depleted mice reconstituted with T lymphocytes to enhance the antibody response in vitro

Cells in culture				Response to SRBC (PFC/culture)
	Spleen		Peritoneal cells	
Unfractionated	Nonadherent	Adherent		
Normal	-	-	-	2120 ± 97
CP	-	-	-	5986 ± 226
TXB	-	-	-	376 ± 56
TXB-CP	-	-	-	420 ± 42
TXB-T	-	-	-	1990 ± 95
TXB-T-CP	-	-	-	4750 ± 230
-	Normal	Normal	-	1710 ± 66
-	Normal	CP	-	5026 ± 128
-	Normal	TXB	-	1570 ± 93
-	Normal	TXB-CP	-	1750 ± 51
-	Normal	TXB-T	-	1766 ± 54
-	Normal	TXB-T-CP	-	3920 ± 72
-	Normal	-	Normal	1720 ± 50
-	Normal	-	CP	5056 ± 175
-	Normal	-	TXB	1350 ± 66
-	Normal	-	TXB-CP	1410 ± 87
-	Normal	-	TXB-T	1906 ± 113
-	Normal	-	TXB-T-CP	4030 ± 210
TXB	-	Normal	-	936 ± 90
TXB	-	-	Normal	1886 ± 50
-	TXB	Normal	-	670 ± 48
-	TXB	-	Normal	1800 ± 45

Mice used as cell donors are designated as follows: Normal - untreated; CP - C. parvum treated; TXB - thymectomized irradiated bone marrow reconstituted; XB - irradiated bone marrow reconstituted; T - reconstituted with purified T lymphocytes.

4:9 Role of recruitment and proliferation in macrophage stimulation

It has been shown that C. parvum induces not only proliferation of macrophages in the liver but also causes proliferation of macrophage precursors from an extra-hepatic source (Warr and Šljivić, 1974). The C. parvum induced macrophage proliferation was found to be largely responsible for the increase of phagocytic activity in vivo (Warr, 1973). In order to determine whether T cell dependent activation of macrophages after administration of C. parvum is dependent on their recruitment and proliferation, the following experiment was performed. Mice were irradiated with a dose of 800 R and reconstituted within 6 hours with either nylon wool column purified T cells or unfractionated spleen cells. These animals were treated with C. parvum 2 days later and their spleens removed 7 days after irradiation for use in culture. Adherent spleen cells or peritoneal cells were used to supplement nonadherent cells from untreated animals in cultures. The results showing the PFC response of cultures containing various cell combinations are given in Table 23. It can be seen that macrophages from irradiated mice treated with C. parvum failed to enhance the PFC response and that irradiation did not affect the supporting functions of macrophages from otherwise untreated animals. Reconstitution of irradiated mice with purified T cells restored the ability of the macrophages to be activated by C. parvum. This restoration was complete in the case of splenic macrophages and partial in the case of peritoneal cells. Similar results were obtained with cells from mice reconstituted with spleen cells and treated with C. parvum except that this time peritoneal cells enhanced the PFC response more than did adherent spleen cells.

From this, it can be concluded that for the enhancement of the in vitro immune response, there is no need for either recruitment of macrophages from extra-splenic sources or proliferation of macrophages in the spleen. Also of interest was the compartmentalization effect when comparing the reconstitution with T cells or whole spleen cells. T cells

Table 23. Lack of recruitment and proliferation of macrophages during their stimulation by C. parvum for the enhancement of the in vitro antibody response

Cells in culture				Response to SRBC (PFC/culture)
	Spleen		Peritoneal cells	
Unfractionated	Nonadherent	Adherent		
Normal	-	-	-	1940 ± 44
C. parvum	-	-	-	5390 ± 142
-	Normal	Normal	-	1860 ± 108
-	Normal	C. parvum	-	4066 ± 134
-	Normal	Irradiated	-	1540 ± 73
-	Normal	X-C. parvum	-	1660 ± 147
-	Normal	X-T	-	1846 ± 80
-	Normal	X-T-C. parvum	-	3676 ± 56
-	Normal	X-S	-	1536 ± 110
-	Normal	X-S-C. parvum	-	2476 ± 204
-	Normal	-	Normal	2000 ± 86
-	Normal	-	C. parvum	4830 ± 93
-	Normal	-	Irradiated	1776 ± 109
-	Normal	-	X-C. parvum	1796 ± 123
-	Normal	-	X-T	1986 ± 163
-	Normal	-	X-T-C. parvum	2560 ± 172
-	Normal	-	X-S	1796 ± 114
-	Normal	-	X-S-C. parvum	4400 ± 226

Mice used as cell donors are designated as follows: Normal - untreated; C. parvum - C. parvum treated; X - irradiated; T - reconstituted with purified T lymphocytes; S - reconstituted with spleen cells. Mice were reconstituted with cells on the day of irradiation and were given C. parvum 2 days later. Cells for culture were harvested 5 days after C. parvum.

affected the response of splenic macrophages whereas spleen cells seem better able to restore the peritoneal cell compartment.

4:10 Effect of culturing macrophages on their ability to enhance the in vitro antibody response

From the preceding sections, it could be seen that macrophages from C. parvum treated animals were capable of enhancing the in vitro immune response of untreated nonadherent cells to SRBC. There was a possibility that this enhancing capacity of pretreated macrophages diminished with time. To test this hypothesis, adherent spleen cells and peritoneal cells from both normal and C. parvum pretreated animals were cultured alone in Marbrook vessels for either 3 or 5 days. At the end of this time, the so-called primary culture period, the cells were supplemented with nonadherent cells from either normal or C. parvum pretreated animals. The results are shown in Table 24. Here it can be seen that even normal macrophages did not respond as well when cultured alone for a period of time. Reconstituting normal nonadherent cells with adherent spleen cells or peritoneal cells from C. parvum animals showed an enhanced activity in comparison to normal cells but this enhancement was lower when using adherent spleen cells cultured for 5 days when compared to those cultured for 3 days. This enhancement was also lower than one normally expected if C. parvum adherent spleen cells used to reconstitute normal nonadherent cells without any prior culturing. In general the enhancing ability of C. parvum pretreated adherent spleen cells seemed greater than that of pretreated peritoneal cells.

Therefore a period of prior culture of macrophages from C. parvum treated animals showed that they were still able to enhance the in vitro antibody response. However this enhancement was not as great as with freshly obtained cells and did show a decrease with the length of time for which the cells were precultured.

4:11 Attempts to elicit C. parvum enhancement totally in vitro

It was attempted to elicit the enhancement due to C. parvum totally

Table 24. Effect of culturing macrophages on the maintenance of their ability to enhance the in vitro antibody response of nonadherent cells to SRBC

Primary culture		Secondary culture	PFC/culture (Anti-SRBC)	
Adherent cells	Peritoneal cells	Nonadherent spleen cells	Duration of primary culture	
			3 days	5 days
Normal	-	Normal	1230 ± 49	1340 ± 100
Normal	-	*C. parvum*	1540 ± 81	1390 ± 10
-	Normal	Normal	1200 ± 180	1480 ± 100
-	Normal	*C. parvum*	1630 ± 49	1550 ± 71
C. parvum	-	Normal	2380 ± 199	1940 ± 160
C. parvum	-	*C. parvum*	2656 ± 170	1980 ± 100
-	*C. parvum*	Normal	2340 ± 380	1630 ± 10
-	*C. parvum*	*C. parvum*	1970 ± 91	1630 ± 150

10^6 adherent spleen cells or peritoneal cells from both untreated or *C. parvum* pretreatment mice were cultured alone for either 3 or 5 days. At the end of this time 14 x 10^6 normal nonadherent cells or 13 x 10^6 *C. parvum* pretreated nonadherent cells were added along with 2 x 10^6 SRBC to the cultures. This phase termed the 'secondary culture' was for the normal period of 4 days after which time the response to SREC was assayed by the usual method.

in vitro. The protocol of this experiment was that 20 x 10^6 spleen cells were cultured for either one or two days in the presence of 100 µg or 10 µg *C. parvum* (dry weight of organisms). This type of *C. parvum* was specially prepared for *in vitro* use as it had been killed by heat inactivation rather than by formalin treatment. Other cultures were set up on day 0 with either 100 µg or 10 µg *C. parvum* present. SRBC (2 x 10^6) were added on day 0 to all the cultures. Then the normal culture time was allowed (4 days), after which the cultures were assayed for their response to SRBC. The results are shown in Table 25. It can be seen that preculturing the cells for 2 days without any antigen reduced the antibody response to 35% of its normal level. This was presumably due to cell death being greater in cultures that were not stimulated by the presence of antigen. The presence of 10 µg *C. parvum* in culture for whatever length of time depressed the antibody response. The only apparent enhancement was observed when 100 µg *C. parvum* was added to culture on day 0 but on statistical analysis the enhancement proved not to be significant at the 5% level.

4:12 *Effect of the addition of C. parvum in vitro on the ability of different cell populations to respond to SRBC in vitro*

In view of the work of people like Mackaness (1971) who found that to observe macrophage activation *in vivo* it was necessary to have T cells present, it was always intriguing that nonadherent cells from *C. parvum* pretreated animals had no effect on normal adherent cells. Nonadherent cells from *C. parvum* treated animals were cultured with normal adherent cells in the presence of various doses of *C. parvum*. The results are shown in Table 26. Under these conditions it was possible to obtain an enhancement when *C. parvum* nonadherent cells were cultured with normal adherent cells in the presence of 100 µg *C. parvum*. This enhancement was comparable to that of reconstituting normal nonadherent cells with *C. parvum* adherent cells. As the dose of *C. parvum* present in culture was reduced so the antibody response was reduced and doses of 10 and 1 µg

Table 25. Effect of addition of C. parvum in vitro (two different doses) on the in vitro response to SRBC

Spleen cells in culture	Dose of C. parvum (µg)	PFC/culture (Anti-SRBC)		
		Number of days preculture with C. parvum		
		2	1	0
Unfractionated	Nil	710 ± 62	2170 ± 137	2150 ± 96
Unfractionated	10	630 ± 37	736 ± 28	1200 ± 88
Unfractionated	100	1110 ± 79	1000 ± 127	2836 ± 265[a]

20×10^6 spleen cells were cultured for either 1 or 2 days in the presence of either 100 or 10 µg C. parvum (dry weight of organisms). Other cultures were set up on day 0 containing either 100 or 10 µg C. parvum. 2×10^6 SRBC were added to all cultures on day 0.
a = P is > 0.05

Table 26. Effect of addition of C. parvum in vitro on the ability of different cell populations to respond to SRBC in terms of in vitro antibody synthesis

Spleen cells in culture			Dose of C. parvum	PFC/culture
Unfractionated	Nonadherent	Adherent	in culture (μg)	Anti-SRBC
Normal	-	-	Nil	2560 ± 167
C. parvum	-	-	Nil	6270 ± 228
-	C. parvum	Normal	Nil	1996 ± 128
-	Normal	C. parvum	Nil	4636 ± 269
-	C. parvum	Normal	100	4276 ± 172
-	C. parvum	Normal	50	1896 ± 146
-	C. parvum	Normal	10	1210 ± 181
-	C. parvum	Normal	1	910 ± 253

Cell types and numbers used in culture as follows:
Unfractionated = 20 x 10^6 cells/culture; nonadherent = 13 x 10^6 cells/culture; adherent = 10^6 cells/culture;
Normal = spleen cells from untreated animals; C. parvum = spleen cells from animals pretreated with C. parvum.

Table 27. Effect of preculturing with C. parvum in vitro for various times on the ability of different cell populations tc respond to SRBC in vitro

Spleen cells in culture		Dose of C. parvum (µg)	PFC/culture (Anti-SRBC)		
			Number of days preculture with C. parvum		
Nonadherent	Adherent		2	1	0
C. parvum	Normal	100	1320 ± 81	2040 ± 158	4820 ± 271
C. parvum	Normal	50	860 ± 79	1860 ± 123	3400 ± 145
C. parvum	Normal	10	286 ± 25	870 ± 154	2290 ± 200
C. parvum	Normal	1	336 ± 62	740 ± 50	1670 ± 175

Cell types and numbers used in culture:
Nonadherent = 15×10^6 cells/culture; Adherent = 10^6 cells/culture; Normal = cells from untreated animals; C. parvum = cells from animals pretreated with C. parvum 5 days previously.
Adherent and nonadherent cells were cultured for either 1 or 2 days in the presence of various concentrations of C. parvum in vitro. On day 0 2×10^6 SRBC were added to all cultures.

C. parvum in fact depressed the response.

The final attempt to use C. parvum in vitro was to culture C. parvum nonadherent cells with normal adherent cells and various doses of C. parvum for 1 or 2 days before the addition of antigen. SRBC were added to all cultures on day 0. Cultures were also set up on day 0 which contained C. parvum adherent cells, normal nonadherent cells, SRBC and various doses of C. parvum. The results are shown in Table 27. Here in vitro presensitization of the cells with C. parvum did not lead to a greater enhancement of the in vitro antibody response, in fact it caused a depression of the response. Though the higher the dose of C. parvum, the higher the antibody response. There was also a difference when the results of Tables 26 and 27 are compared with regard to the response of cells given C. parvum in vitro on day 0. In Table 26, any dose other than 100 µg C. parvum present in the cultures gave a lower response than even the normal controls, but in Table 27 doses of both 100 µg and 50 µg both enhanced the response to levels above those normally expected of normal controls. Before any firm conclusions are drawn from these experiments it is necessary to repeat these experiments to establish consistent experimental data.

4:13 Double Marbrook chambers and C. parvum

The finding in earlier experiments that lymphocytes from animals sensitized to C. parvum can enhance the performance of normal macrophages in the presence of C. parvum in vitro suggested the idea of culturing C. parvum pretreated and normal cells in double Marbrook chambers. The initial protocol was as follows:- 3×10^6 spleen cells from C. parvum pretreated mice were cultured with both 3×10^5 SRBC and 100 µg C. parvum in the inner chamber. Other cultures were set up with one or the other or neither of these additions. The outer chamber contained 30×10^6 normal spleen cells and 3×10^6 SRBC. The two cell suspensions were separated by a cell impermeable membrane. The results are shown in Table 28. From this it can be seen that when normal spleen cells were cultured

Table 28. The effect of culturing C. parvum pretreated spleen cells and normal spleen cells separated by a cell impermeable membrane in double Marbrook culture vessels on the in vitro antibody response to SRBC

Contents of inner chamber	Contents of outer chamber	Anti-SRBC response of cells in outer chamber	
		PFC/culture	PFC/10^6 cells
C. parvum spleen cells + SRBC	Normal spleen cells + SRBC	3946 ± 447	132 ± 14
C. parvum spleen cells + *C. parvum*	Normal spleen cells + SRBC	3150 ± 373	105 ± 12
C. parvum spleen cells + *C. parvum* + SRBC	Normal spleen cells + SRBC	6060 ± 279	202 ± 9
C. parvum spleen cells	Normal spleen cells + SRBC	2870 ± 207	96 ± 7
Nil	Normal spleen cells + SRBC	2956 ± 188	99 ± 6
Nil	*C. parvum* spleen cells + SRBC	7126 ± 469	238 ± 16

Cell number and antigen dose:
In inner chamber - 3×10^6 *C. parvum* spleen cells, 100 µg *C. parvum* and/or 3×10^5 SRBC.
In outer chamber - 30×10^6 normal or *C. parvum* spleen cells and 3×10^6 SRBC.

Table 29. Effect of culturing C. parvum pretreated cell populations and normal spleen cells separated by a cell impermeable membrane in double Marbrook culture vessels on the in vitro antibody response to SRBC

Contents of inner chamber	Contents of outer chamber	Anti-SRBC response of cells in outer chamber	
		PFC/culture	PFC/10^6 cells
C. parvum spleen cells + *C. parvum* + SRBC	Normal spleen cells + SRBC	5440 ± 403	181 ± 13
C. parvum nonadherent cells + *C. parvum* + SRBC	Normal spleen cells + SRBC	2400 ± 157	80 ± 5
C. parvum nonadherent cells + *C. parvum* adherent cells + *C. parvum* + SRBC	Normal spleen cells + SRBC	3500 ± 82	117 ± 3
Nil	Normal spleen cells + SRBC	2998 ± 129	100 ± 4
Nil	*C. parvum* spleen cells + SRBC	8791 ± 423	293 ± 14

Cell number and antigen dose:
In inner chamber - 3×10^6 *C. parvum* spleen cells; 2×10^6 *C. parvum* nonadherent cells; 10^5 *C. parvum* adherent cells; 100 µg *C. parvum* and 3×10^5 SRBC.
In outer chamber - 30×10^6 normal or *C. parvum* spleen cells and 3×10^6 SRBC.

in the outer chamber and the inner chamber contained C. parvum pretreated spleen cells together with SRBC and C. parvum, the response of the normal cells to SRBC was enhanced. This enhancement did not occur with any of the other cell combinations in the inner chamber. As the cells were separated by a cell impermeable membrane, it can be postulated that the enhancement observed was due to some soluble factor(s). A further experiment utilising Marbrook double chambers was performed in which both unfractionated and fractionated C. parvum spleen cell populations were cultured in the inner chamber with 3×10^5 SRBC and 100 µg C. parvum. The results are shown in Table 29. Here again an enhancement of the SRBC response of normal cells in the outer chamber was observed when the inner chamber contained whole spleen cells from animals pretreated with C. parvum together with C. parvum and SRBC. This enhancement could be abolished if the inner chamber contained only nonadherent cells from C. parvum pretreated animals. The enhancement could be partially restored by recombining C. parvum nonadherent and adherent cells in the inner chamber. Whatever the mechanism of this enhancement, it is important to note that if the outer chamber contained 30×10^6 normal cells cultured with 1.5 µg DNP-KLH and the inner chamber contained 3×10^6 C. parvum pretreated cells together with 3×10^5 SRBC and 100 µg C. parvum, the response to DNP-KLH was enhanced; results shown in Table 30. These were merely preliminary experiments and much further investigation of the phenomenon described here is needed.

Table 30. Effect of culturing C. parvum pretreated cells and normal spleen cells separated by a cell impermeable membrane in double Marbrook culture vessels on the in vitro antibody response to DNP-KLH

Contents of inner chamber	Contents of outer chamber	Anti-DNP response of cells in outer chamber	
		PFC/culture	PFC/10^6 cells
C. parvum spleen cells + C. parvum + SRBC	Normal spleen cells + DNP-KLH	4400 ± 301	147 ± 10
Nil	C. parvum spleen cells + DNP-KLH	3999 ± 394	133 ± 13
Nil	Normal spleen cells + DNP-KLH	609 ± 29	20 ± 8

Cell number and antigen dose:
In inner chamber - 3×10^6 C. parvum spleen cells + 100 µg C. parvum + 3×10^5 SRBC.
In outer chamber - 30×10^6 normal or C. parvum spleen cells and 1.5 µg DNP-KLH.

DISCUSSION

A summary of the findings of this section can be listed thus:-

1. Pretreating mice with C. parvum led to an enhancement of the in vitro antibody response of their spleen cells to SRBC.

2. This enhancement was maximal at 5 to 7 days after treatment and led to an in vitro response 2.5 to 3 times that of the normal anti-SRBC response. Even at 21 days after C. parvum pretreatment spleen cells showed an enhanced antibody response.

3. C. parvum pretreatment did not affect the in vitro response to DNP-POL.

4. C. parvum pretreatment caused normal unprimed spleen cells to give an in vitro response to DNP-KLH.

5. C. parvum pretreatment did not alter the kinetics of the in vitro anti-SRBC response.

6. By means of cell fractionation and irradiation, this enhancement was found to mediated by macrophages.

7. T cells were found to be required for the induction of macrophages capable of this enhancement.

8. Recruitment of macrophages from neither extra-splenic precursors nor proliferation of splenic macrophages was needed for this enhancement of the in vitro anti-SRBC response.

9. Macrophages cultured for either 3 or 5 days from C. parvum treated animals were still capable of enhancing the in vitro anti-SRBC response.

10. Addition of C. parvum to normal cells in vitro did not cause any significant enhancement of the in vitro anti-SRBC response.

11. Addition of 100 µg C. parvum on day 0 to nonadherent cells from C. parvum pretreated animals and normal adherent cells caused an enhancement of the in vitro anti-SRBC response.

12. Experiments using double Marbrook vessels indicated the possible presence of a factor(s) produced by C. parvum pretreated cells which was

capable of mediating this enhancement of the in vitro anti-SRBC response.

This section was a study of the effects of pretreating mice with C. parvum on the in vitro immune response of their spleen cells. It has been previously shown that systemic pretreatment of mice with C. parvum led to an adjuvant effect as demonstrated by the response of these pretreated animals to both T independent antigens (Howard et al., 1973a,b) and T dependent antigens (Warr and James, 1975). Previous work had provided evidence that this involves stimulation of B cells at a time when cell mediated immunity, but not helper activity, of T cells was inhibited. It has been suggested that this adjuvant effect was mediated by activated macrophages (Howard et al., 1973a,b). Using the Marbrook system, it has been possible to show an in vitro enhancement of the antibody response to SRBC, a thymus dependent antigen (Claman and Chaperon, 1969), but not to DNP-POL, a thymus independent (Diener et al., 1970) and a macrophage independent antigen (Feldmann and Basten, 1972). This is at variance with the in vivo results of Howard et al. (1973a,b). It is possible that had the kinetics of the response of these pretreated cells to DNP-POL been studied they would have been affected, i.e. maybe the response to DNP-POL would have been prolonged. This study of the adjuvant effect in vitro is different from that of Maillard and Bloom (1972) who using Bordetella pertussis and PPD were only able to obtain an enhancement of the in vitro SRBC response when these adjuvants were present in the cultures of spleen cells from mice primed previously with either B. pertussis or tubercule bacilli.

The adjuvant effect was associated with adherent cells and not with the nonadherent cell population. These adherent cells were radioresistant providing strong evidence that they were macrophages (Roseman, 1969; Sjöberg et al., 1972). However there are reports that T cells become radioresistant after priming (Campbell and Cooper, 1975) and also it is possible to separate T memory cells, responsive to ovalbumin, by their adherence to glass wool (Durkin et al., 1975). There appears, however,

to be no evidence for a population of T cells that are both radiation resistant and adherent to glass. Experiments, to be reported later, using carrageenan pretreatment of mice would seem to support the idea that it is the adherent cell population in the form of macrophages that is affected by C. parvum pretreatment and is able to mediate the enhanced antibody response to SRBC in vitro. Experiments with irradiated cells also suggest that C. parvum pretreatment did not confer the property of glass adherence upon the radiosensitive cells (lymphocytes) nor conversely did it alter the radiosensitivity of the nonadherent cell population.

The results of the experiments employing TXB mice show that similar to the in vivo (Allison and Davies, 1971) and the in vitro (Maillard and Bloom, 1972) situations, the adjuvant effect needs the presence of T cells for its development. Further to the effect of C. parvum on T cells, it was found that animals unprimed to KLH gave a response in vitro to DNP-KLH after being pretreated with C. parvum. Whether this is a direct activation of T cells by C. parvum or an indirect effect mediated by macrophages is open to debate. If C. parvum has a direct enhancing effect on T cells then why is it not possible to obtain an enhancing effect on the anti-SRBC response using nonadherent cells from C. parvum treated animals in combination with normal adherent cells? It is possible, however, that when animals are pretreated with C. parvum the effect on T cells is of short duration and so taking spleen cells from animals 5 days after C. parvum means that any effect on the lymphocytes cannot be observed. It is possible to postulate that priming with C. parvum has a transient effect on T cells causing them to elaborate a factor(s) which is capable of affecting the macrophages. Once however macrophages have been stimulated they are capable of mediating enhanced in vitro responses to SRBC for some time. This is suggested by the finding that spleen cells from C. parvum pretreated animals gave an enhanced response to SRBC up to 21 days after pretreatment.

Several points of an experimental nature arise when discussing the

experiments using TXB mice. Firstly, macrophages from XB mice pretreated with C. parvum did not give the same degree of enhancement as those from untreated mice. It is possible that this was due to incomplete restoration of the T cell compartment of lymphocytes after irradiation, as unprimed T cells have been shown to be radiation sensitive (Mitchell and Miller, 1968; Anderson et al., 1974; Sprent et al., 1974). Secondly, when TXB spleen cells were reconstituted with normal peritoneal cells they gave a higher response to SRBC than when reconstituted with normal adherent spleen cells. This suggests that peritoneal cells contain T cells, in agreement with Raff and Owen (1971).

Experiments trying to induce the enhancement caused by C. parvum totally in vitro using normal unfractionated spleen cells were not successful. This is to be contrasted with the work of Murgo and Athanassiades (1975). Using Bordetella pertussis vaccine they found that it was possible to obtain an enhanced SRBC response in normal mouse spleen cells merely by adding the vaccine to their Mishell-Dutton cultures. Addition of the vaccine was also capable of enhancing the antibody response of spleen cells which had been depleted of either T cells or adherent cells. They found that B. pertussis, per se released into the culture medium a soluble component(s) that was adjuvant in its activity. They suggest that one of the ways in which B. pertussis acts as an adjuvant is by direct stimulation of the PFC precursors. It can be argued as in the discussion of Chapter Three that any factor, provided it is dialysable, produced in a Marbrook culture is diluted out because of the differences in the volume of culture fluid used in the Marbrook system as compared to the Mishell-Dutton system or that the adjuvant activity of C. parvum is not mediated by the same mechanisms. It was, however, possible to obtain an enhanced antibody response to SRBC if nonadherent cells from C. parvum pretreated animals were cultured with normal adherent cells and 100 µg C. parvum. This can be explained if it is postulated that on priming with C. parvum the effect on T cells is short lived and needs to be reactivated by the

presence of further C. parvum. This will be discussed more fully in the general discussion when a model of macrophage activation will be attempted.

As shown here, C. parvum activation of macrophages requires the presence of T cells. It is possible that this activation is mediated through a T cell factor. Experiments using double Marbrook chambers suggest that C. parvum pretreated cells in the presence of both SRBC and 100 µg C. parvum produce a soluble factor(s) capable of enhancing the antibody response of normal cells to SRBC. This factor was not produced when adherent cells were removed from the cultures of C. parvum pretreated spleen cells. The specificity of this factor could not be assessed because, though it could potentiate the response of unprimed cells to DNP-KLH, the design of this experiment was not satisfactory. As DNP-KLH is a soluble antigen, it will obviously diffuse through the nucleopore membrane and so it will also be present in the top chamber. Therefore, though it is perfectly feasible to postulate the production of nonspecific factors, the possibility of the production of a specific factor(s) which would potentiate the response to DNP-KLH cannot be ruled out.

The requirement for T cells which has been demonstrated necessitates that it is postulated that the continuous presence of activated T cells is not required and that activation of macrophages is not possible if sensitized T cells are not reactivated in vitro with the same antigen. However, there are various pieces of evidence that C. parvum has an adjuvant effect in the absence of T cells. C. parvum had an adjuvant effect on the SRBC response in vivo in T cell deprived mice and also on the response to pneumococcal polysaccharide (Howard et al., 1973b), but not on the response to SRBC in nude mice (Warr and James, 1975). Also, C. parvum stimulated the phagocytic activity in T cell deprived mice (Woodruff et al., 1974) and generated macrophages cytotoxic to tumour cells in both these and nude mice (Ghaffar et al., 1975).

However C. parvum mediates its effects in vivo, the experiments using macrophages from animals that had been irradiated and then recon-

stituted with either T cells or spleen cells suggest that the ability of macrophages to enhance the *in vitro* antibody response to SRBC was independent of their need to divide or to mobilize macrophage precursors from the bone marrow. It is known that both of these functions are radiation sensitive (Warr and Šljivić, 1974;

Recent work by Wiener (1975) has shown that peritoneal macrophages from *C. parvum* treated mice were better able to reconstitute the anti-SRBC response of normal nonadherent cells than normal peritoneal cells. She and Bandieri (Wiener and Bandieri, 1975) have also shown that peritoneal macrophages from *C. parvum* pretreated animals handled the antigen KLH differently from the peritoneal cells of normal animals. Their initial uptake of antigen was slower and the degree of degradation was less. They suggest that intensification of the presentation of antigen to lymphocytes on the macrophage surface could be one of the ways in which *C. parvum* elicits its adjuvant effects. It is interesting to note that when antigen handling in Biozzi high and low responder mice was studied, macrophages from high responder strains showed the same pattern of antigen handling as did the *C. parvum* macrophages (Wiener and Bandieri, 1974).

Relevant to the need for T cells in the activation of macrophages by *C. parvum* is the work of Christie and Bomford. They found that *in vitro* mouse peritoneal cells could not be activated by direct exposure to *C. parvum*, activation in their system being measured by the inhibition of DNA synthesis of leukaemic cells. By mixing *C. parvum* and spleen cells from *C. parvum* immune mice with the normal cells, they were able to achieve activation. This activation could be abolished by treating the cells with anti-theta and complement. Supernatants from these pretreated cells incubated with *C. parvum* were also capable of activating normal macrophages (Christie and Bomford, 1975). *In vivo* experiments (Bomford and Christie, 1975) showed that a second injection of *C. parvum* accelerated the development of spleen cells capable of mediating the activation of normal macrophages. Spleen cells from T cell depleted mice showed a

reduced capacity to mediate the activation of normal macrophages *in vitro*. They did, however, find no difference in the course of macrophage activation or of splenomegaly in T cell depleted mice. They suggest that this is because either macrophage activation at the early stages is the result of the direct interaction of macrophages and *C. parvum* or that *C. parvum* is able to mobilize the residual T cell population so efficiently as to avoid detectable changes in the rate of macrophage activation.

With regard to the effect of *C. parvum* pretreatment on the response to DNP-POL *in vitro*, it was found never to enhance the response to this antigen. This is to be compared with the report by Zola (1975) that *C. parvum per se* is a B cell mitogen and that for the expression of this mitogenicity macrophages are required. If this is the case, one would expect that pretreatment of the animals with *C. parvum* would also cause the response to DNP-POL to be enhanced. Howard *et al.* (1973b) found that *C. parvum* pretreatment enhanced the response to pneumococcal polysaccharide. This is a thymus independent antigen as is DNP-POL. It is possible that *C. parvum* pretreatment effected the response to DNP-POL but that the right parameters for detecting such alterations were not applied and so any changes were missed.

A *non sequitur* resulting from the effects of *C. parvum* on the *in vitro* response to SRBC is that the possible production of IgG by these cultures was never assayed. If, as it appears, T cells are somehow involved in this activation of macrophages and the production of IgG is also known to be highly T cell dependent (Taylor and Wortis, 1968), it is possible that *C. parvum* pretreated spleen cells in culture will produce IgG antibody as well as the IgM which has been assayed in all these responses.

These then are some of the specific points of discussion concerning this section. It is hoped to produce a model of macrophage activation in the final chapter which is testable and will encompass some of these findings.

CHAPTER FIVE - THE EFFECT OF *MYCOBACTERIUM LEPRAEMURIUM* INFECTION ON THE ANTIBODY RESPONSE

INTRODUCTION

One of the fascinating characteristics of human leprosy is the spectrum of the disease (Ridley and Jopling, 1966), presumably due to the innate resistance varying from patient to patient. At the high resistance end of the spectrum are patients with pure tuberculoid leprosy (TL) who often have only a single lesion and in which no bacilli are found. The granuloma contain a few foci of activated macrophages. At the opposite end of the spectrum are patients with lepromatous leprosy (LL). Here the granuloma contain histiocytes and undifferentiated macrophages, containing vast numbers of bacilli. In between these two extremes are the patients with intermediate forms of the disease.

In a series of elegant studies by Closs and his co-workers, murine leprosy has been studied as a model for the spectral aspect of human leprosy. The causative organism, M. lepraemurium, is an obligate intracellular parasite, with a generation time of 8-10 days and is nontoxic to the cells in which it multiplies. Stefansky in 1903 described two types of infection, the glandular form involving lymph nodes alone, and the musculocutaneous form in which the animals suffered from a disseminated infection. Kawaguchi (1959) presented results that suggested that susceptibility to subcutaneous inoculation with M. lepraemurium was different in different inbred strains. The work of Closs started with outbred mice inoculated intravenously with M. lepraemurium and he observed that the infection progressed at different rates in different animals (Closs and Haugen, 1973). From this observation, they went on to compare the response of outbred mice to that of various inbred mouse strains. In the outbred strain, the variation in host response was more marked after small doses of M. lepraemurium than after large doses. Mice of the same inbred strain showed the same degree of infection but there were marked interstrain differences. The types of infection produced in the strains C3H and C57/Bl were found to represent the polar forms of murine leprosy (Closs and Haugen, 1974). They found that infection became disseminated

in C3H mice which were unable to mount a delayed type hypersensitivity reaction against M. lepraemurium antigens. C57/Bl mice on the other hand seemed to be able to localize the infection and produce a delayed hypersensitivity response to M. lepraemurium antigens. C57 mice also developed increased resistance to reinfection whereas C3H did not (Closs and Haugen, 1975). C3H mice produced more antibodies than C57/Bl mice against M. lepraemurium antigens though this was less marked with larger inocula (Closs and Kronvall, 1975).

Other work on the immunological status of mice infected with M. lepraemurium has suggested evidence that the disease is associated with disturbances of CMI. Examples include prolongation of skin graft survival across strong histocompatibility barriers (Ptak et al., 1970), blockade of the induction of allergic encephalomyelitis (Bullock et al., 1971) and impairment of sensitization to haptens (Ptak et al., 1970). Another parallel between murine and human leprosy is found in the pathology of the respective lymphoid organs. The paracortical areas of lymph nodes from patients with lepromatous leprosy are swamped by granulomatous infiltrates that appear to displace the population of small lymphocytes normally present (Turk and Waters, 1971). In murine leprosy not only are the paracortical areas of the lymph nodes replaced by histiocytes but the splenic white pulp is also invaded (Bullock, 1974).

The course of infection in CBA mice is that they begin to die after 16 to 20 weeks when given 10^9 organisms intravenously. However this is dose dependent i.e. the greater the dose the shorter the interval between infection and subsequent death. An antibody response to M. lepraemurium antigens can be detected 5 to 8 days after infection but this diminishes with time after infection. The appearance of delayed hypersensitivity is about 4 weeks after the start of infection though this yet again is dose dependent. All these data are either observations made in the course of experiments not included in this thesis or personal communications from I.N. Brown.

As it is known that the causative organism of mouse leprosy grows in cells of the MPS, it was decided to look at the effect of infection on the *in vivo* and *in vitro* response to antigen. The antigens used were SRBC for both *in vivo* and *in vitro* experiments and DNP-POL and DNP-Ficoll in *in vitro* experiments only.

RESULTS

As described in the introduction, Mycobacterium lepraemurium, the rodent leprosy bacillus, is an obligate intracellular dweller and is found particularly inside the cells of the macrophage series. However, in terminal stages of infection other cell types may be affected. Systemically infected mice characteristically show macrophages overloaded with bacilli, ever increasing numbers of granulomata and increasing spleno- and hepatomegaly (Šljivić and Brown, unpublished results). Therefore the effect of M. lepraemurium on both the in vivo and in vitro antibody responses was studied.

5:1 In vivo antibody response to SRBC of mice infected with M. lepraemurium

Mice infected intravenously with 10^9 M. lepraemurium organisms 4, 7, 11 and 14 weeks previously plus uninfected controls were injected with either 5×10^6 or 10^9 SRBC intravenously. Four days later, the spleens were removed and the number of antibody producing cells (PFC) present in the spleen of each mouse was determined using the Cunningham method. The results are shown in Table 31 and are expressed as $\log_{10}$PFC/spleen, PFC/spleen, geometric mean ($\times 10^{-3}$) and PFC/10^6 spleen cells. Firstly, it can be seen that as the infection progressed the response to both doses of SRBC was depressed. This depression was very marked at 7 weeks post-infection in the case of the lower dose of SRBC and by 14 weeks post-infection the response to both doses was very much depressed. One interesting point was that animals challenged with 10^9 SRBC 4 weeks post-infection showed a slightly enhanced response to SRBC. However, this enhancement was not significant at the 5% level.

5:2 The in vitro antibody response of spleen cells from M. lepraemurium infected animals

Standard Marbrook cultures were performed using spleen cells from animals at various stages of M. lepraemurium infection. The results are presented in a series of tables (Tables 32 to 41) and a summary of the

Table 31. In vivo antibody response to SRBC in CBA mice infected with M. lepraemurium

Weeks after infection	Dose of SRBC injected					
	5×10^6 SRBC			10^9 SRBC		
	Log_{10}	Geometric mean ($\times 10^{-3}$)	PFC/10^6 spleen cells	Log_{10}	Geometric mean ($\times 10^{-3}$)	PFC/10^6 spleen cells
Uninfected controls	5.85 ± 0.02	703	6051 ± 391	5.97 ± 0.04	937	4899 ± 452
4	5.82 ± 0.06	667	3016 ± 831	6.01 ± 0.04	1015	3545 ± 725
7	5.33 ± 0.02	216	729 ± 73	5.97 ± 0.02	930	2997 ± 241
11	5.33 ± 0.06	214	605 ± 100	5.76 ± 0.07	570	1250 ± 154
14	4.87 ± 0.16	75	204 ± 83	5.28 ± 0.03	189	446 ± 21

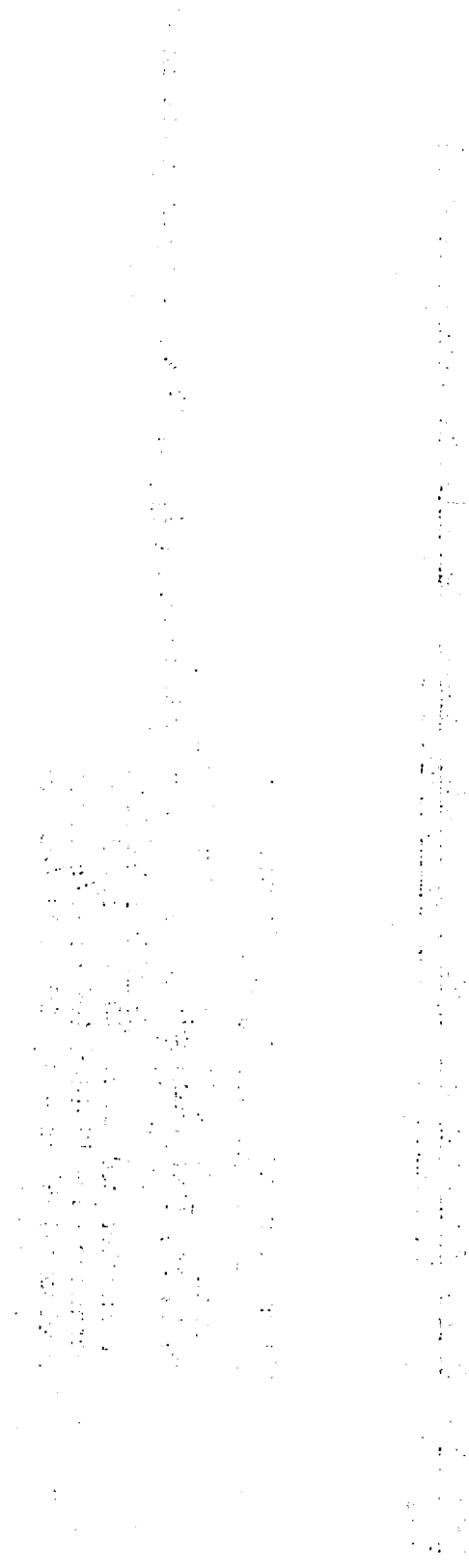


Table 32. Part I of preliminary results using spleen cells from M. lepraemurium infected mice

Cell numbers and types are designated thus:

Normal = spleen cells from uninfected animals;
4, 7, 11, 14 week = spleen cells from animals at 4, 7, 11 and 14 weeks after infection with M. lepraemurium;
Unfractionated = 20 x 10^6 cells/culture
Nonadherent = 12-14 x 10^6 cells/culture
Peritoneal cells = 4 x 10^6 cells/culture

Table 32. Part I of preliminary results using spleen cells from M. lepraemurium infected mice (spleens from 4, 7, 11 and 14 week stages of infection)

Spleen cells in culture			Peritoneal	PFC/culture
Unfractionated	Nonadherent	Adherent	Cells	Anti-SRBC
Normal	-	-	-	2086 ± 142
4 week	-	-	-	4690 ± 324
7 "	-	-	-	1976 ± 238
11 "	-	-	-	1246 ± 114
14 "	-	-	-	290 ± 78
-	Normal	-	Normal	2140 ± 85
-	4 week	-	Normal	2000 ± 90
-	7 "	-	Normal	1750 ± 117
-	11 "	-	Normal	1790 ± 78
-	14 "	-	Normal	1466 ± 33
-	Normal	-	4 week	7146 ± 299
-	Normal	-	7 "	5300 ± 307
-	Normal	-	11 "	1860 ± 86
-	Normal	-	14 "	2010 ± 190
-	Normal	-	-	190 ± 12
-	4 week	-	-	116 ± 28
-	7 "	-	-	140 ± 48
-	11 "	-	-	96 ± 46
-	14 "	-	-	80 ± 34

Table 33. Part II of preliminary studies using spleen cells from M. lepraemurium infected mice

See legend for Table 32.

Except

8, 11, 15, 18 week = spleen cells from animals 8, 11, 15 and 18 weeks after infection with M. lepraemurium.

Table 33. Part II of preliminary studies using spleen cells from M. lepraemurium infected mice (spleens from 8, 11, 15 and 18 week stages of infection)

Spleen cells in culture			Peritoneal	PFC/culture
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC
Normal	-	-	-	2140 ± 82
8 week	-	-	-	1846 ± 90
11 "	-	-	-	960 ± 57
15 "	-	-	-	620 ± 22
18 "	-	-	-	230 ± 24
Normal	-	-	Normal	2600 ± 10
8 week	-	-	Normal	2100 ± 95
11 "	-	-	Normal	1716 ± 51
15 "	-	-	Normal	1510 ± 59
18 "	-	-	Normal	1410 ± 66
-	Normal	Normal	-	1886 ± 41
-	8 week	Normal	-	1946 ± 42
-	11 "	Normal	-	1800 ± 26
-	15 "	Normal	-	1706 ± 61
-	18 "	Normal	-	1290 ± 53
-	Normal	8 week	-	1686 ± 34
-	Normal	11 "	-	1192 ± 64
-	Normal	15 "	-	610 ± 13
-	Normal	18 "	-	230 ± 35

findings is shown graphically in Figs. 3 and 4. Tables 32 and 33, the first two in the series, show the results of the preliminary experiments using spleen cells from infected animals in the in vitro response to SRBC. These cells showed a biphasic response to SRBC, at weeks 4 and 6 of infection the response to SRBC was enhanced but by 11 weeks post-infection the response was depressed and this depression became more marked with time after infection. In these preliminary experiments, various cell fractionation and recombination experiments were carried out. It can be seen that the response of non-adherent cells from infected animals can be almost fully restored by the addition of either normal spleen adherent or normal peritoneal cells. This restoration was not as efficient when non-adherent cells from 14 and 18 weeks of infection were used. If normal non-adherent spleen cells were reconstituted with adherent cells from various stages of infection, the anti-SRBC response was both enhanced and depressed - the enhancement being mediated by adherent spleen cells from early stages of infection and the depression by adherent cells from late stages of the infection. However, if peritoneal cells from infected animals were used, the response to SRBC was enhanced at 4 and 7 weeks post-infection and then returned to a normal level. This was at a time when the adherent cells from these animals were suppressing the response. If normal peritoneal cells were added to unfractionated spleen cells at various stages of infection it was possible to greatly enhance the response of cells from infected animals when they would normally give a depressed response to SRBC i.e. at late stages of infection.

After the preliminary experiments, the responses of spleen cells from infected animals were tested at various times after infection. These results are shown in Tables 34 to 41. The response to SRBC was enhanced 2 to 3 times that of the normal response at week 4 of infection (Table 36) but by week 10 there was a significant depression of the response (Table 39). The response to DNP-POL was unaffected until week

Fig. 3. The in vitro antibody response of spleen cells from M. lepraemurium infected mice

Closed circles represent the response of spleen cells from M. lepraemurium infected animals to SRBC.

Open triangles represent the response of spleen cells from M. lepraemurium infected animals to DNP-POL.

Range of normal responses to SRBC and DNP-POL lie between the solid and dashed lines respectively.

Fig. 4. Effect of fractionation and reconstitution of spleen cells from M. lepraemurium and normal animals on the in vitro response to SRBC

Range of normal response to SRBC lies between the solid lines.

FIG.3

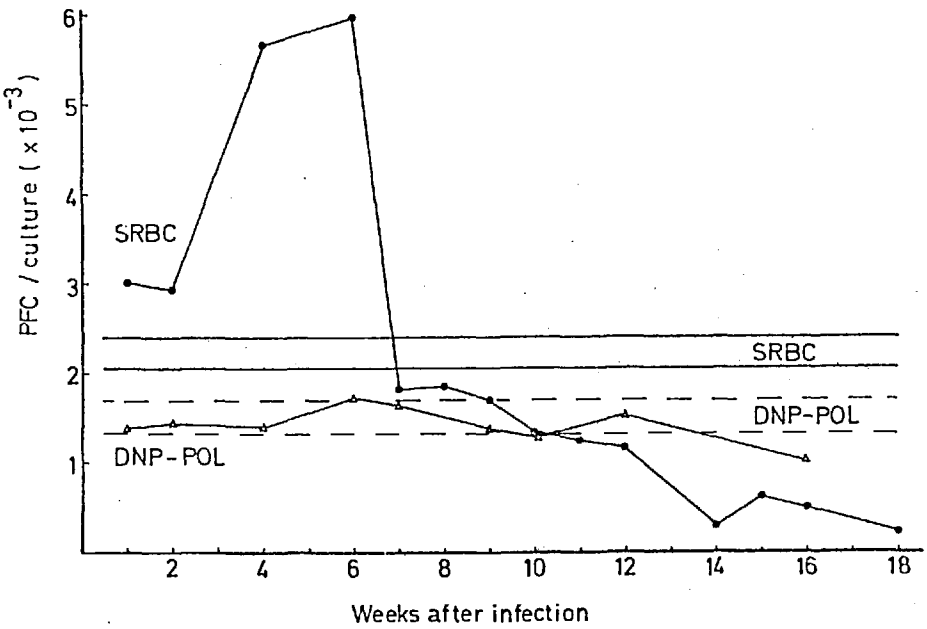
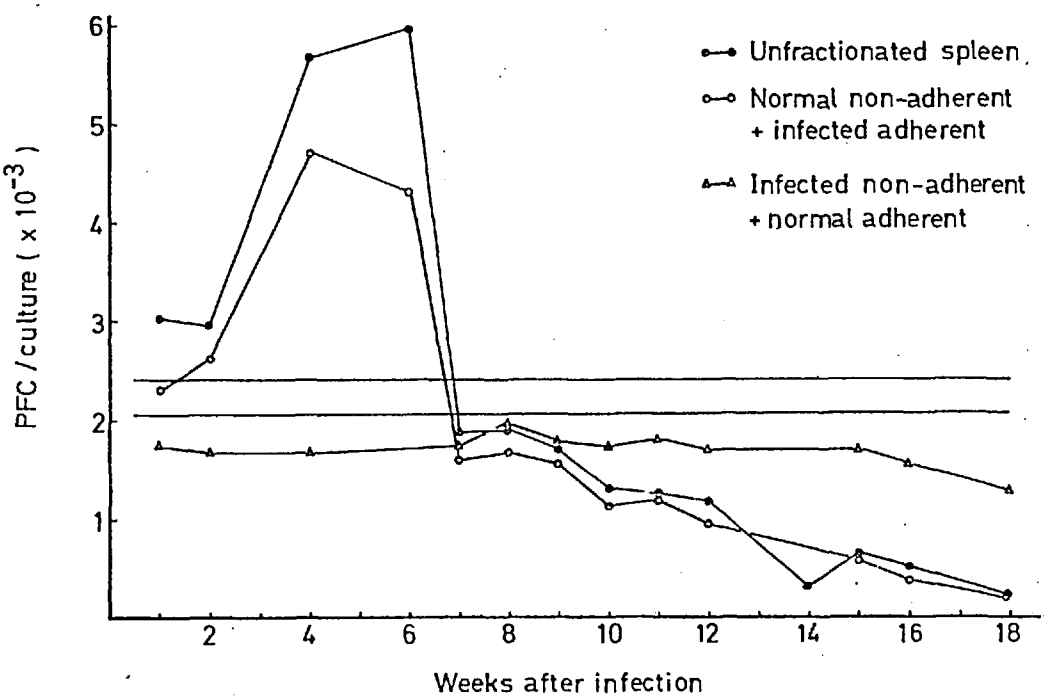

PFC / culture (x 10^{-3})
6
5
4
3
2
1
SRBC
SRBC
DNP-POL
DNP-POL
2
4
6
8
10
12
14
16
18
Weeks after infection

FIG.4


PFC / culture (x 10^{-3})
6
5
4
3
2
1
Unfractionated spleen
Normal non-adherent + infected adherent
Infected non-adherent + normal adherent
2
4
6
8
10
12
14
16
18
Weeks after infection

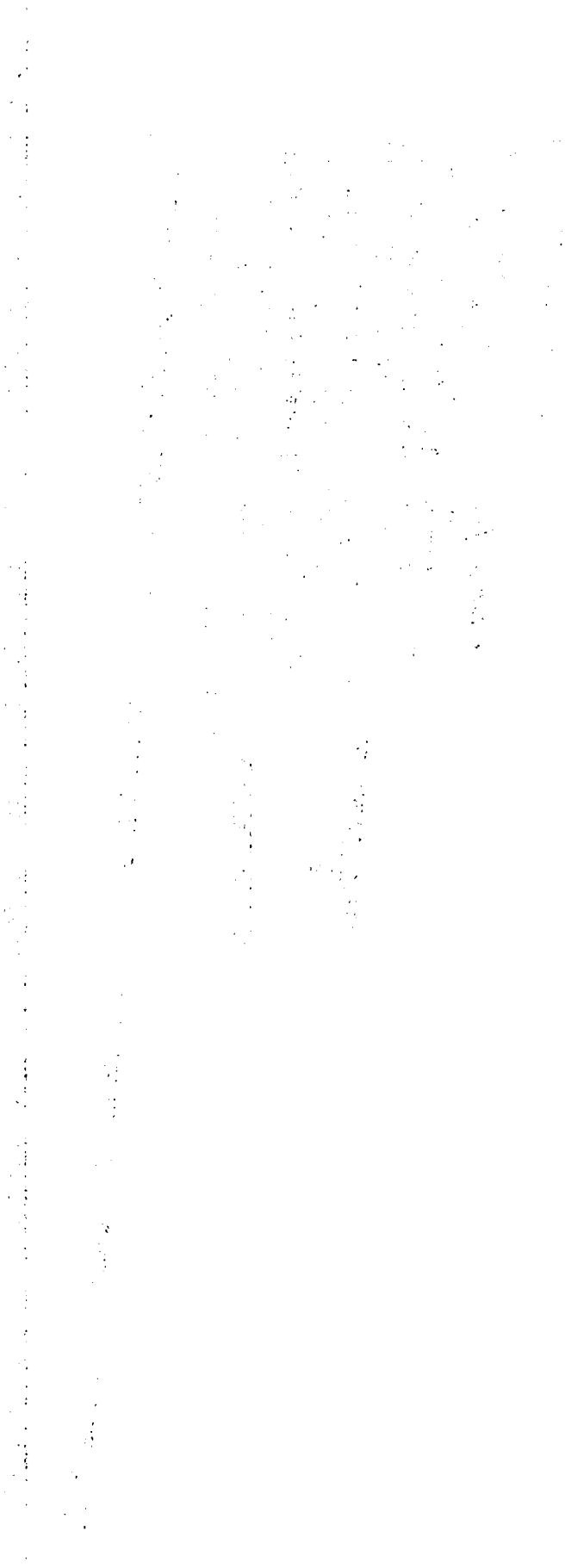


Table 34. In vitro response of spleen cells from mice 1 week after infection with M. lepraemurium to SRBC and DNP-POL

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2216 ± 168	1376 ± 56
Mlm	-	-	-	3020 ± 105	1376 ± 95
-	Normal	-	-	150 ± 31	1400 ± 115
-	Mlm	-	-	146 ± 36	1496 ± 45
-	Normal	Mlm	-	2306 ± 128	1260 ± 39
-	Mlm	Normal	-	1740 ± 91	1526 ± 82
Normal	-	-	Normal	2426 ± 84	1500 ± 64
Mlm	-	-	Normal	3160 ± 145	1416 ± 143
Normal	-	-	Mlm	2890 ± 55	1350 ± 72
Mlm	-	-	Mlm	2100 ± 166	1476 ± 53
-	Normal	-	Normal	1860 ± 109	1466 ± 81
-	Mlm	-	Normal	1916 ± 54	1390 ± 26
-	Normal	-	Mlm	2086 ± 104	1350 ± 87
-	Mlm	-	Mlm	2076 ± 53	1446 ± 91

Table 34. In vitro response of spleen cells from mice 1 week after infection with M. lepraemurium to SRBC and DNP-POL

Cell type and number are designated thus:

Normal = spleen cells from uninfected mice;
Mlm = spleen cells from mice infected with M. lepraemurium;
Unfractionated = 20 x 10^6 cells/culture
Nonadherent = 12-14 x 10^6 cells/culture
Adherent = 10^6 cells/culture
Peritoneal cells = 10^6 cells/culture.

This applies to Tables 35 to 41 as well.

Table 35. In vitro response of spleen cells from mice 2 weeks after infection with M. lepraemurium to SRBC and DNP-POL

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2190 ± 104	1330 ± 70
Mlm	-	-	-	2946 ± 104	1460 ± 83
-	Normal	-	-	70 ± 25	1516 ± 62
-	Mlm	-	-	126 ± 34	1470 ± 90
-	Normal	Mlm	-	2616 ± 143	1770 ± 108
-	Mlm	Normal	-	1670 ± 61	1466 ± 36
Normal	-	-	Normal	2300 ± 59	1480 ± 112
Mlm	-	-	Normal	2850 ± 211	1390 ± 146
Normal	-	-	Mlm	3156 ± 91	1420 ± 70
Mlm	-	-	Mlm	3198 ± 99	1448 ± 59
-	Normal	-	Normal	1806 ± 87	1490 ± 67
-	Mlm	-	Normal	1956 ± 96	1460 ± 70
-	Normal	-	Mlm	2386 ± 100	1436 ± 22
-	Mlm	-	Mlm	2350 ± 103	1556 ± 71

Table 36. In vitro response of spleen cells from mice 4 weeks after infection with M. lepraemurium to SRBC and DNP-PO

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2096 ± 114	1570 ± 54
Mlm	-	-	-	5666 ± 131	1400 ± 117
-	Normal	-	-	150 ± 31	1606 ± 41
-	Mlm	-	-	190 ± 30	1470 ± 97
-	Normal	Mlm	-	4686 ± 386	1446 ± 113
-	Mlm	Normal	-	1680 ± 50	1440 ± 79
Normal	-	-	Normal	2596 ± 104	1564 ± 139
Mlm	-	-	Normal	5470 ± 178	1346 ± 87
Normal	-	-	Mlm	5360 ± 210	1456 ± 166
Mlm	-	-	Mlm	6596 ± 229	1806 ± 126
-	Normal	-	Normal	1826 ± 105	1336 ± 116
-	Mlm	-	Normal	1710 ± 91	1526 ± 61
-	Normal	-	Mlm	4810 ± 82	1436 ± 67
-	Mlm	-	Mlm	5236 ± 102	1360 ± 79

Table 37. In vitro response of spleen cells from mice 7 weeks after infection with M. lepraemurium to SRBC and DNP-POL

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2056 ± 125	1566 ± 64
Mlm	-	-	-	1820 ± 134	1660 ± 59
-	Normal	-	-	156 ± 30	ND
-	Mlm	-	-	120 ± 22	ND
-	Normal	Mlm	-	1600 ± 67	ND
-	Mlm	Normal	-	1736 ± 43	ND
Normal	-	-	Normal	2660 ± 88	ND
Mlm	-	-	Normal	2716 ± 62	ND
Normal	-	-	Mlm	3616 ± 133	ND
Mlm	-	-	Mlm	3780 ± 91	ND
-	Normal	-	Normal	1736 ± 68	ND
-	Mlm	-	Normal	1580 ± 42	ND
-	Normal	-	Mlm	2246 ± 63	ND
-	Mlm	-	Mlm	2260 ± 75	ND

Table 38. In vitro response of spleen cells from mice 9 weeks after infection with M. lepraemurium to SRBC and DNP-PO

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherert	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2155 ± 93	1416 ± 103
Mlm	-	-	-	1706 ± 51	1366 ± 87
-	Normal	-	-	150 ± 31	1390 ± 81
-	Mlm	-	-	110 ± 24	1336 ± 88
-	Normal	Mlm	-	1586 ± 41	1496 ± 59
-	Mlm	Normal	-	1730 ± 74	1400 ± 70
Normal	-	-	Normal	2610 ± 78	1520 ± 89
Mlm	-	-	Normal	2196 ± 79	1500 ± 63
Normal	-	-	Mlm	2060 ± 119	1420 ± 74
Mlm	-	-	Mlm	2116 ± 70	1356 ± 91
-	Normal	-	Normal	1786 ± 87	1320 ± 101
-	Mlm	-	Normal	1780 ± 108	1340 ± 95
-	Normal	-	Mlm	1686 ± 42	1386 ± 39
-	Mlm	-	Mlm	1730 ± 76	1516 ± 74

Table 39. In vitro response of spleen cells from mice 10 weeks after infection with M. lepraemurium to SRBC and DNP-FO

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2390 ± 87	1386 ± 37
Mlm	-	-	-	1306 ± 49	1300 ± 74
-	Normal	-	-	100 ± 18	ND
-	Mlm	-	-	146 ± 59	ND
-	Normal	Mlm	-	1120 ± 76	ND
-	Mlm	Normal	-	1720 ± 50	ND
Normal	-	-	Normal	2986 ± 152	ND
Mlm	-	-	Normal	1990 ± 58	ND
Normal	-	-	Mlm	1040 ± 93	ND
Mlm	-	-	Mlm	880 ± 41	ND
-	Normal	-	Normal	1904 ± 47	ND
-	Mlm	-	Normal	1566 ± 86	ND
-	Normal	-	Mlm	900 ± 88	ND
-	Mlm	-	Mlm	836 ± 50	ND

Table 40. In vitro response of spleen cells from mice 12 weeks after infection with M. lepraemurium to SRBC and DNP-POL

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2316 ± 151	1716 ± 54
Mlm	-	-	-	1176 ± 80	1536 ± 70
-	Normal	-	-	160 ± 68	ND
-	Mlm	-	-	126 ± 25	ND
-	Normal	Mlm	-	956 ± 76	ND
-	Mlm	Normal	-	1700 ± 50	ND
Normal	-	-	Normal	3066 ± 54	ND
Mlm	-	-	Normal	2020 ± 96	ND
Normal	-	-	Mlm	1790 ± 123	ND
Mlm	-	-	Mlm	1226 ± 84	ND
-	Normal	-	Normal	1766 ± 87	ND
-	Mlm	-	Normal	1640 ± 75	ND
-	Normal	-	Mlm	1216 ± 47	ND
-	Mlm	-	Mlm	940 ± 91	ND

Table 41. In vitro response of spleen cells from mice 16 weeks after M. lepraemurium infection to SRBC and DNP-POL

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2410 ± 179	1520 ± 62
Mlm	-	-	-	500 ± 50	1036 ± 76
-	Normal	-	-	160 ± 29	ND
-	Mlm	-	-	46 ± 26	ND
-	Normal	Mlm	-	380 ± 42	ND
-	Mlm	Normal	-	1546 ± 64	ND
Normal	-	-	Normal	2706 ± 183	ND
Mlm	-	-	Normal	1680 ± 70	ND
Normal	-	-	Mlm	2076 ± 147	ND
Mlm	-	-	Mlm	500 ± 80	ND
-	Normal	-	Normal	1606 ± 75	ND
-	Mlm	-	Normal	1410 ± 89	ND
-	Normal	-	Mlm	650 ± 62	ND
-	Mlm	-	Mlm	426 ± 84	ND

Table 42. In vitro background to SRBC of spleen cells from normal and M. lepraemurium infected animals

Weeks post infection	Anti-SRBC background*	
	Normal spleen cells	Infected spleen cells
1	80 ± 41	95 ± 61
2	110 ± 23	131 ± 48
4	94 ± 17	80 ± 29
7	114 ± 49	91 ± 19
9	71 ± 19	120 ± 64
10	130 ± 64	75 ± 29
12	64 ± 3	61 ± 43
16	109 ± 34	53 ± 39

* Background response was assayed by culturing spleen cells in vitro for 4 days without antigen (SRBC) being present.

16 of infection (Table 41). Splenic adherent cells from infected animals when used to reconstitute normal nonadherent cells were found to enhance the response at 4 weeks and 6 weeks post-infection and cause a depression at 10 weeks and onwards of the response to SRBC. Peritoneal cells from infected animals were also capable of enhancing the response of normal nonadherent cells to SRBC at 4 weeks post-infection but they also enhanced the response of normal nonadherent cells at 7 weeks when the splenic adherent cells from such animals were only able to restore the response to normal levels. The addition of normal peritoneal cells to unfractionated spleen cells from late stages of infection was able to abrogate the depression of the SRBC response until 16 weeks post-infection when the addition of normal peritoneal cells only caused a partial restoration of the response. Conversely the addition of peritoneal cells from M. lepraemurium infected animals never affected the response of unfractionated normal spleen cells, except for one case at week 10 of infection when the response to SRBC was found to be depressed. Whether this was a cultural artefact or not can be argued but at later stages of infection this depressive effect was not observed. Table 42 is a summary of the background responses to SRBC of both normal and infected spleen cells during the course of the infection. It can be seen that the background response to SRBC of infected spleen cells was never above that of the normal controls. Therefore any enhancement of the response was not due to cross reaction between M. lepraemurium and SRBC.

5:3 T cell dependence of macrophage activation of cells from the early stages of M. lepraemurium infection

In view of reports that in other systems, macrophage activation is T cell dependent (Mackaness, 1971) and results presented in this thesis concerning C. parvum, the early stages of M. lepraemurium infection were investigated. It was postulated that the early enhancement of the antibody response to SRBC in vitro mediated by activated macrophages was T cell dependent.

Table 43. In vitro response of spleen cells from T cell depleted mice infected 6 weeks previously with M. lepraemurium

Spleen cells in culture			Peritoneal	PFC/culture	
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC	Anti-DNP
Normal	-	-	-	2065 ± 90	1605 ± 38
Mlm	-	-	-	5970 ± 189	1730 ± 93
Mlm TXB	-	-	-	425 ± 102	1550 ± 93
Mlm XB	-	-	-	2890 ± 342	1735 ± 113
-	Normal	Normal	-	1750 ± 98	ND
-	Normal	Mlm	-	4265 ± 319	ND
-	Normal	Mlm TXB	-	1610 ± 73	ND
-	Normal	Mlm XB	-	2740 ± 125	ND
-	Normal	-	Normal	1900 ± 141	ND
-	Normal	-	Mlm	5575 ± 270	ND
-	Normal	-	Mlm TXB	1765 ± 137	ND
-	Normal	-	Mlm XB	3435 ± 175	ND

Normal = untreated mice;
Mlm = M. lepraemurium infected mice;
TXB = thymectomized, X-irradiated, bone marrow reconstituted mice;
XB = X-irradiated, bone marrow reconstituted mice.

Thymectomized, X-irradiated, bone marrow reconstituted (TXB) mice and X-irradiated, bone marrow reconstituted (XB) mice plus controls were infected with M. lepraemurium and after 6 weeks the mice were killed and their spleens removed for use in culture. The results are shown in Table 43. Again the response of spleen cells from infected mice showed an enhanced response to SRBC. TXB mice infected with M. lepraemurium showed a decrease in their response to SRBC presumably due to the lack of T cells. XB mice infected with M. lepraemurium did not show as great an enhancement of the anti-SRBC response as did untreated, infected controls. The response to DNP-POL was unaffected by all these treatments.

Spleen cells were fractionated into adherent and nonadherent cell populations and the adherent cell fraction from all 4 groups of mice was reconstituted with normal nonadherent cells. It can be seen that macrophages from infected animals were capable of enhancing the response to SRBC but that macrophages from TXB infected animals were not able to give this enhancement though they were able to restore the response to normal levels. Adherent cells from XB infected mice also gave an enhanced response, but it was not as great as that of untreated infected mice.

Normal nonadherent spleen cells were also reconstituted with peritoneal cells from the various groups of animals. Again the same pattern was found. Peritoneal cells from infected mice gave an enhanced response to SRBC; those from TXB infected mice though capable of reconstituting the response did not enhance it and peritoneal cells from XB infected mice also enhanced the response, though not as much as peritoneal cells from untreated, infected animals. If, however, the enhancement effected by adherent spleen cells and peritoneal cells of XB infected mice is compared it can be seen that peritoneal cells from such mice gave a greater enhancement than did adherent spleen cells.

5:4 Effect of different doses of M. lepraemurium given at different times on the in vitro response to SRBC

Mice were infected at 2, 4 and 6 weeks with 10^3, 10^6 or 10^9 M. lepraemurium

Table 44. Effect of different doses of M. lepraemurium given at different time intervals on the in vitro response to SRBC and DNP-Ficoll

No. of organisms injected	Time post injection (weeks)	PFC/culture	
		Anti-SRBC	Anti-DNP
10^3	2	2520 ± 145	1686 ± 67
	4	3376 ± 216	1586 ± 101
	6	4606 ± 229	1676 ± 75
10^6	2	3000 ± 188	1586 ± 67
	4	4910 ± 304	1600 ± 134
	6	6570 ± 209	1540 ± 41
10^9	2	3366 ± 154	1690 ± 66
	4	6090 ± 58	1580 ± 71
	6	7180 ± 97	1730 ± 31
Nil	0	2190 ± 99	1700 ± 78

murium organisms prior to the removal of their spleens for use in culture. The response to both SRBC and DNP-Ficoll was assayed in vitro. The results are shown in Table 44. The response to SRBC was enhanced in all cases except when using cells from mice given 10^3 organisms 2 weeks before. This enhancement was greater the larger the inoculum of organisms given and also the greater the length of time allowed to elapse between infection and culturing of the cells in vitro. The response to DNP-Ficoll was unaffected at all times no matter what dose of organisms were given or the time after infection when the spleen cells were cultured.

DISCUSSION

The results of this section can be summarised thus:-

1. Infection with M. lepraemurium leads to a depression of the in vivo immune response to SRBC in all cases.

2. At 4 to 6 weeks post-infection the in vitro response to SRBC was enhanced. This enhancement was followed by a depression of the response which by 16 to 18 weeks was almost background level.

3. The in vitro response to DNP-POL was unaffected until late stages of infection, when it was somewhat reduced.

4. The degree of enhancement of the in vitro antibody response was dependent on the dose of M. lepraemurium used to infect the animals.

5. Both the enhancement and the depression of the in vitro SRBC response were mediated by macrophages.

6. T cells were needed to bring about this macrophage enhancement at early stages of infection.

7. Peritoneal cells from infected animals were also capable of enhancing the in vitro SRBC response and remained capable of this enhancement for longer than splenic macrophages.

8. No evidence could be found for the production of a suppressive factor(s) by macrophages in the later stages of infection.

It can be seen that the effect of M. lepraemurium infection on the in vitro antibody response is biphasic. The initial enhancement of the in vitro SRBC response is followed by a suppression. This suppression causes the SRBC response to fall to approximately background levels. The enhancement was mediated by macrophages and needed the presence of T cells thus resembling the enhancement caused by C. parvum pretreatment.

In the analysis of the later stages of M. lepraemurium infection, it was found that there was a depression of the in vitro anti-SRBC response which was also mediated by macrophages. The possible role of a macrophage factor or direct contact of infected macrophages being the cause(s) of the depression was ruled out because the addition of normal peritoneal cells

to unfractionated spleen cells late in infection (11 to 14 weeks) reconstituted the response. These findings were supported by those of Favila and Jimenez (1975). Using the Mishell-Dutton system, they found that the in vitro response to SRBC was depressed in late stages of infection and that the in vivo response was hardly above background levels at 5 months of infection. They could not demonstrate any serum factor(s) present in the serum of infected animals capable of suppressing the in vitro response to SRBC of normal animals and they therefore concluded that there was an intrinsic defect in the function of one or several of the cell types involved in the response to SRBC. The depression of the response may be due to a variety of factors. Firstly, overloading of macrophages with mycobacteria may interfere with their ability to ingest, process and present antigen in the normal manner (Kolsch, 1970). A second possibility is that antibody production against M. lepraemurium which can be detected 4 to 8 days after infection (results not shown here) causes the formation of antigen-antibody complexes which bind to the macrophage surface thus blocking sites for the binding of T cell factors required for the antibody response.

Contrary to the results shown here, Ptak et al. (1970) showed that mice infected with M. lepraemurium had impaired cell mediated immune responses such as delayed rejection of grafts and the depression or absence of contact sensitivity. However, the humoral responses to bovine serum albumin and SRBC remained normal. They suggested that there was a defect in T cell function of infected mice. This is obviously in conflict with the findings in this section but it must be said that Ptak was measuring the humoral response to these antigens whereas here the PFC response was measured and this is a more sensitive technique. Results obtained by Šljivić and Brown (unpublished results) also showed that there was no depression of the response to oxazolone even at 11 weeks post-infection when the PFC response was very much depressed. In this series of experiments, they also assessed the ^{51}Cr-SRBC uptake as a

measure of the phagocytic activity of the MPS. They found that the uptake of ^{51}Cr-SRBC was increased in the spleen and liver and was decreased in the lungs and bone marrow of infected animals. This indicated that the phagocytic capabilities of infected animals were enhanced.

When studying the enhancement of the SRBC response at early stages of infection it was found that both splenic adherent cells and peritoneal cells from infected animals could mediate this enhancement. Peritoneal cells however were capable of enhancing the response to SRBC at a later stage of infection than splenic adherent cells. This is probably because mice were systemically infected and so initially most of the bacteria were present in the liver and spleens of these animals and so there was a lag between infection of the liver and the spleen and that of peritoneal cells. No effect of infection can really be noticed until 3 or 4 weeks post-infection. The generation time of the murine leprosy bacillus is between 7 to 10 days depending on the tissue in which it is growing (Brown and Krenzien, 1976) and so it can be imagined that it takes some time before there are sufficient bacteria present to trigger any response. Also, the time of first appearance of the enhanced response to SRBC is at the time of the development of a granulomatous reaction in those strains of mice which are going to respond in this way (Closs and Haugen, 1975). Peritoneal cells have been shown to be better at killing the bacteria (Brown, personal communication), so it would take longer for the infection to invade the peritoneal cells. That this activation of macrophages requires the presence of T cells makes it reminiscent of Mackaness' system for the activation of macrophages (Mackaness, 1971) and of results presented in the previous section about the mechanism of C. parvum activation of macrophages.

During the course of these experiments, the background response to SRBC (in terms of antibody) was never affected by the infection. This is to be noted in view of the findings of Rook (1975). He was looking at the mitogenic responses of mice infected with M. ulcerans and found that

as a consequence of severe infection, the lymph node cells of such mice contained phagocytic cells which caused spontaneous transformation of these cells. This transformation could be inhibited by specific antigen or PHA and eliminated by treatment with carbonyl iron. As he could mimic this effect by the addition of peritoneal cells from infected mice to normal lymph node, thymus or peritoneal lymphocytes, he suggested that this phenomenon was mediated by a factor released by macrophages.

Two technical points to be made are that preparing spleen cell suspensions from these infected animals must be done with the utmost care to maintain the viability of the cells. This is often difficult due to the splenomegaly resulting from infection which makes the spleens so large as to be difficult to manipulate. Also, all these experiments were done using live bacilli and it would have been interesting to see the effects of killed bacilli on the in vitro response so as to compare the effects more meaningfully with those of C. parvum.

Throughout this study of the effect of M. lepraemurium infection on the in vitro response, lymphocyte performance remained normal as assessed by: firstly, the response of unfractionated spleen cells to DNP-POL which was normal until late stages of infection suggesting that B cells maintained their level of responsiveness; secondly, the fact that it was possible to reconstitute the response to SRBC of nonadherent cells from infected animals by the addition of normal splenic adherent cells or peritoneal cells, suggesting that T cell helper activity, alongside B cell function, remained unaffected. The finding that the in vivo response to oxazolone was not impaired (Šljivić and Brown, personal communication) supports the conclusion about the maintenance of normal T cell function during the period of infection under study. The depression of the DNP-POL response at later stages of infection can possibly be explained if one imagines that the macrophages are so overloaded with bacilli that there is not a single macrophage uninfected. If what Lee et al. (1976) have found is true that antigens classically thought of as macrophage

independent require a small number of functional macrophages, then this terminal suppression of the response to DNP-POL may reflect this fact. However, as the response to oxazolone and the response of nonadherent cells from infected animals to SRBC when reconstituted with normal adherent cells are both depressed at late stages on infection, this explanation is not sufficient. It is possible that this overall depression reflects a stage in infection when cells other than macrophages become invaded by bacteria and hence the overall depression of these functions at this stage of infection.

CHAPTER SIX - OTHER INFECTIONS AND THEIR EFFECTS ON THE *IN VITRO* ANTIBODY RESPONSE

Bacille Calmette-Guerin (BCG)

BCG is an attenuated strain of Mycobacterium bovis routinely used to vaccinate against tuberculosis in man. BCG unlike M. lepraemurium, is a facultative intracellular bacteria rather than an obligate one. Collins (1971) in a study on the immunogenicity of various mycobacteria found that after systemic infection BCG multiplied mainly in the lungs of normal mice so that there was a 2 to 3 log. increase in the viable bacterial count over a period of 30 days. There was a limited amount of growth in the spleen with a subsequent decline from day 14, indicating a developing immune response in this organ. There was no early growth in the liver and after 2 weeks the number of viable bacteria in this organ also declined. The mice first showed significant tuberculin hypersensitivity at about day 14. Three months after infection the lungs of these mice contained 10^4 viable organisms and the liver and spleen 10^3 viable organisms. It has also been shown that it is not necessary for an organism to increase in vivo in terms of viable numbers in order that an immune response can be mounted against it, but that it must be able to persist in the MPS for some time (Collins and Mackaness, 1970). This is also true for infection with salmonellae.

BCG is similar to C. parvum in that it is capable of suppressing experimental tumours in animals (Old et al., 1959; Zbar et al., 1971) and has been used in immunotherapy of human malignancies (Mathe, 1971; Baker and Taub, 1973). A series of papers by Mackaness and his co-workers have attempted to analyze the immunopotentiation in terms of cell proliferation, SRBC response and the modulation of the response to tumour antigens brought about by BCG. They found that a fully viable inoculum of BCG caused blast transformation in the regional lymph node cells and that the rate of cell division was proportional to the log. dose of viable organisms given. They were also able to distinguish differences in immunogenicity in various BCG preparations which correlated with the virulence of the

strain - the more virulent the strain the greater its immunogenicity (Mackaness et al., 1973). They showed that BCG enhanced both the direct and indirect antibody production which was associated with a delayed type hypersensitivity mediated by T cells. However, this potentiation only occurred when BCG and SRBC reached the same regional lymph node (Miller et al., 1973).

Rojas-Espinosa et al. (1974) have studied the two possible mechanisms by which macrophages could be activated in the tuberculous lesions of rabbits and they found that activation was due to the ingestion and digestion of hydrolyzable material by macrophages at the border of the caseous centres of dermal BCG lesions. However, activation due to immunological mechanisms (lymphocytes, lymphokines and antigenic products of bacteria) were demonstrated in the macrophages located more peripherally in the non-necrotic tissue of the lesions. Here again one can construct a Mackaness-like activation pathway. T cells are sensitized to specific antigen and produce lymphokines which in turn stimulate the macrophage membrane and so trigger it to become an activated macrophage. There is a report of the production of cytophilic antibody which can bind to the macrophage surface (Svejcar et al., 1972). On the basis of the involvement of the MPS in BCG infection it was decided to investigate its effects on the in vitro antibody response.

Salmonella typhimurium

Mouse typhoid is a naturally occurring infection which provides a model of Salmonella infections in man. In this role it has been used to investigate the herd immunity (Greenwood et al., 1931), the genetic (Gowen, 1960) and the nutritional aspects (Schneider, 1965) of host resistance. However, the mechanisms of acquired resistance to the organism has been a point of contention for a while. Rowley and his fellow workers have for a long time held the point of view that immunity is due to specific opsonic antibodies (Rowley et al., 1964). In contrast, Mackaness believes that CMI leading to the production of activated macrophages is

important (Blanden et al., 1966). An interesting point to note is that resistance to reinfection by microbial agents seems to be connected with how long they remain in the animal. In a study by Collins et al. (1966), it was found that S. enteritidis (virulent strain - LD_{50} 5 x 10^2-10^3) which produces an infection indistinguishable from that caused by S. typhimurium, and S. gallinarum (avirulent - LD_{50} 8 x 10^6) were present in the mouse for periods of longer than a week whereas S. pullorum (avirulent at 10^8 organisms) was eliminated from the tissues within 3 days. Animals infected primarily with S. enteritidis or S. gallinorum were completely resistant to reinfection with S. enteritidis whereas those initially infected with S. pullorum failed to evoke an effective bactericidal mechanism.

The above led to the classification of salmonellae as facultative intracellular bacteria. However, there is no clear evidence of salmonellae growing in macrophages. There is in fact evidence that the intracellular environment of guinea pig macrophages is unfavourable to the proliferation of some salmonellae (Hsu and Radcliffe, 1968). It has also been shown that the rate of phagocytosis of S. typhimurium by guinea pig macrophages was significantly greater in the presence of specific antiserum and was inversely proportional to the relative virulence of the strains of bacteria (Wells and Hsu, 1970). More recent work by Hsu and Mayo (1973) showed that guinea pig macrophages were capable of inactivating ingested S. typhimurium but previous infection did not increase the bactericidal capacity of the macrophages. Immune macrophages did however clump together in the presence of salmonellae. This they attributed to the presence of cytophilic antibody on the immune macrophages. These antibodies could be eluted and transferred to normal macrophages by incubation in the presence of immune serum. From this they proposed that the presence of cytophilic antibody on immune macrophages represented an expression of antibacterial cellular immunity by enhancing the clumping of these macrophages.

However a Mackaness like system of macrophage activation can be postulated as a means of defence against the invading bacteria. Antibodies may play a role in the acquired resistance to this bacterium but only as an additional aid to CMI. Therefore due to the suggestion that macrophages are involved in the defence against infection by this bacterium, the effects of S. typhimurium infection on the in vitro immune response was studied. The results are presented in this section.

Malaria

There are over 100 species of malaria parasite infecting a wide variety of vertebrate hosts. Mosquitos infect the host through transmission of the infectious stages of the parasite, the sporozite. These invade the hepatic cells and undergo asexual replication, schizogony, to give rise to merozoites. A second cycle of asexual multiplication occurs in the erythrocytes. The merozoite developes into a trophozoite and then into a schizont containing merozoites. When the host red cell has ruptured, these merozoites are able to invade fresh red cells and give rise to new cycles of erythrocytic schizogony. It is at this stage of infection that results in fever, anaemia, splenomegaly and the other symptoms of malaria.

There are 2 malarial parasites which have mice as their host; these are Plasmodium berghei berghei (P. berghei) and Plasmodium berghei yoelii (P. yoelii). P. berghei causes death in most mouse strains whereas P. yoelii causes a self resolving disease.

Humoral immunity is a major feature of acquired resistance to malaria but probably only a small percentage of this is protective at the level of parasitized red cells. Histological examination of the spleens and livers of malarial animals reveals phagocytosis of parasitized and normal red cells (WHO, 1968). Protective antibody operates on the erythrocytic merozoites preventing reinvasion and promoting phagocytosis of mature intra-erythrocytic stages (WHO, 1975). Nude mice infected with P. berghei show high mortality rates and if P. yoelii is used a normally

low grade resolving infection turns into a fulminating parasitaemia (Clark and Allison, 1974). In rats, parasitaemias due to P. berghei were higher in neonatally thymectomized rats than in controls and lasted twice as long with higher mortality rates (Brown et al., 1968). These results seem to suggest that the depletion of T cells eliminates the protective immune response.

Immunosuppression as a result of malarial infection is well established. Lymphocytes from P. berghei infected mice show a decreased number of PHA responsive cells (Jayawardena et al., 1975) in comparison with cells from P. yoelii infected animals whose PHA responsive population remained unaltered. Salaman et al. (1969) reported that primary and secondary antibody responses of mice to SRBC were reduced when the antigen was given during a period of P. yoelii parasitaemia. This was confirmed by Greenwood et al. (1971a) but in the same report they failed to find any impairment of the rejection of skin grafts or other cell mediated responses. In further studies, Greenwood et al. (1971b) found that clearance of colloidal carbon and ^{51}Cr-labelled SRBC was enhanced during the period of maximal parasitaemia and maximal immunosuppression. When the distribution of ^{51}Cr in the liver and spleen was determined after intravenous injection, malarial mice showed an increased radioactive uptake in the liver but the spleens were the same as the controls. They took this to indicate that far from there being an impairment of phagocytic function it was enhanced during the course of malarial infection. If peritoneal cells from malaria infected mice were allowed to ingest SRBC and then given to normal recipients, they induced fewer PFCs than did similar peritoneal cells from normal mice (Loose et al., 1972). This suggested an impairment of handling and processing of antigen by macrophages in malarial infection.

The point of interest is that it is possible that all these infections in some way affect the macrophage. Therefore to analyze this possibility, the effect of such infections on the ability of mouse spleen cells to respond in vitro to both T independent and dependent antigens was investigated.

6:1 BCG

BCG like other Mycobacteria exhibits a slow growth rate both *in vivo* and *in vitro* and has the ability to survive and multiply within normal tissue macrophages (Youmans, 1957). This being the case, preliminary experiments were carried out to see the effect of BCG infection of the *in vitro* antibody responses to both SRBC and DNP-Ficoll.

6:1:1 Time course of the effect of BCG infection on the in vitro antibody response to SRBC and DNP-Ficoll

Mice were injected intravenously with 10^6 BCG organisms at intervals ranging from 1 to 7 weeks before their spleens were removed for use in culture. The results of two such experiments to assess the effect of BCG on the *in vitro* antibody response are shown in Table 45. The response to SRBC was enhanced at 4, 5, 6 and 7 weeks post-infection. The response to DNP-Ficoll was also enhanced during this period though these results were not consistent between experiments. Cell separation and reconstitution experiments were also carried out. These showed that adherent cells from BCG infected mice were able to enhance the response of normal nonadherent cells if taken from animals infected for 3 or more weeks. Nonadherent cells taken from BCG infected animals were however normal in their responses to SRBC when reconstituted with normal adherent cells. These two experiments were regarded as pilot experiments and on the basis that they did show some enhancing effect on the anti-SRBC response by BCG a further experiment was carried out.

6:1:2 Effect of various doses of BCG given at various time intervals on the in vitro antibody response to SRBC and DNP-Ficoll

As it was found that the enhancement due to the injection of 10^6 BCG organisms was not very great, three doses of BCG were used in this experiment. Animals were injected with either 10^6, 10^7 or 10^8 BCG organisms 2 and 4 weeks before the spleens were removed for use in culture. The results are shown in Table 46. In general, this was a more consistent

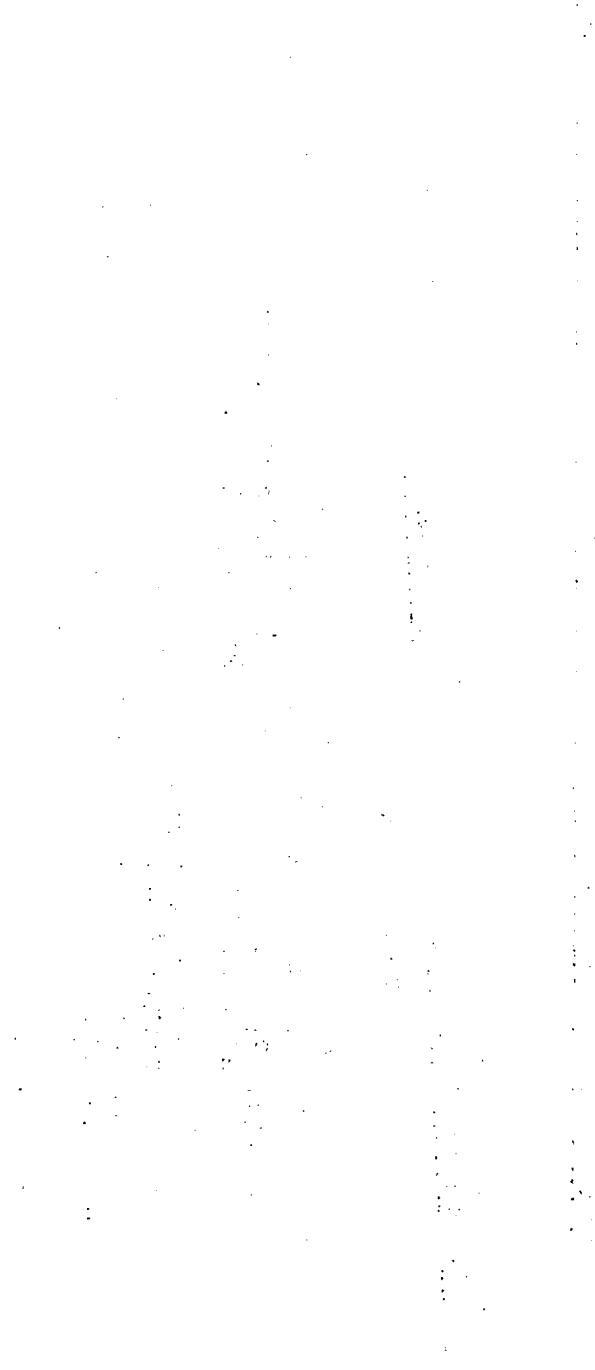


Table 45. Effect of BCG infection on the in vitro response to SRBC and DNP-Ficoll

Cell numbers and types:

Normal = spleen cells from normal mice;
BCG = spleen cells from mice at 1 to 7 weeks after BCG infection;
Nonadherent spleen cells - 14×10^6 cells/culture;
Adherent spleen cells - 10^6 cells/culture.

* P is < 0.005

Table 45. Effect of BCG infection on the in vitro response to SRBC and DNP-Ficoll

Spleen cells in culture			PFC/culture			
			Anti-SRBC		Anti-DNP	
Unfractionated	Nonadherent	Adherent	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Normal	-	-	2170 ± 197	2189 ± 95	1366 ± 81	1750 ± 112
BCG wk 1	-	-	1900 ± 42	ND	ND	1730 ± 71
BCG wk 2	-	-	1880 ± 104	ND	1400 ± 293	ND
BCG wk 3	-	-	2050 ± 83	2200 ± 205	ND	ND
BCG wk 4	-	-	3476 ± 186	2256 ± 258	2056 ± 186*	2196 ± 157
BCG wk 5	-	-	ND	2716 ± 110	ND	2320 ± 193*
BCG wk 6	-	-	3010 ± 71	ND	1856 ± 110*	ND
BCG wk 7	-	-	ND	2660 ± 104	ND	2636 ± 309*
-	Normal	Normal	1810 ± 103	1730 ± 65		
-	Normal	BCG wk 1	1810 ± 93	1676 ± 116		
-	Normal	BCG wk 2	1650 ± 185	ND		
-	Normal	BCG wk 3	2820 ± 100	ND		
-	Normal	BCG wk 4	2760 ± 148	1816 ± 93	ND	
-	Normal	BCG wk 5	ND	2386 ± 104		
-	Normal	BCG wk 6	2810 ± 138	ND		
-	Normal	BCG wk 7	ND	2226 ± 147		
-	BCG wk 1	Normal	1720 ± 209	1746 ± 126		
-	BCG wk 2	Normal	1856 ± 186	ND		
-	BCG wk 3	Normal	1956 ± 85	ND		
-	BCG wk 4	Normal	2080 ± 205	2156 ± 71	ND	
-	BCG wk 5	Normal	ND	1978 ± 208		
-	BCG wk 6	Normal	2016 ± 149	ND		
-	BCG wk 7	Normal	ND	1910 ± 112		

Table 46. Effect of various doses and differing times of BCG infection on the in vitro immune response to SRBC and DNP-Ficoll

Time after BCG infection	Dose of BCG	PFC/culture	
		Anti-SRBC	Anti-DNP
4 weeks	10^6	4010 ± 321	1940 ± 79
	10^7	3390 ± 156	2120 ± 99
	10^8	3986 ± 99	1920 ± 77
2 weeks	10^6	4430 ± 396	1830 ± 96
	10^7	6086 ± 200	1800 ± 82
	10^8	6200 ± 236	1916 ± 74
Controls	Nil	2120 ± 136	2006 ± 56

experiment than the previous two. All doses of BCG enhanced the anti-SRBC response 2 or 3 times that of its normal level. The response to DNP-Ficoll was totally unaffected by the ongoing infection.

6:2 Salmonella typhimurium

This yet again another bacterium whose life cycle involves macrophages. This time the bacterium is killed inside macrophages though this killing is often not very efficient and so bacteria can be found growing in the macrophages of such animals (Blanden et al., 1966).

6:2:1 Effect of the number of S. typhimurium organisms injected and the time of injection on the in vitro response to SRBC and DNP-Ficoll

Mice were injected with 10, 10^3 or 10^6 S. typhimurium organisms subcutaneously 4, 8 and 12 days before their spleens were removed for use in culture. The results are shown in Table 47. It can be seen that, excepting two cases, no matter what dose of organisms was injected nor when they were injected, they caused an increase in the response to SRBC without affecting the DNP-Ficoll response. In the case where both the SRBC and DNP-Ficoll responses were depressed, one can argue that these were a poor set of cultures and so the results of this group of cultures is not valid. This experiment was repeated using the same doses of S. typhimurium only giving them at 1, 3, 5, 7, 14 and 21 days prior to the removal of spleens for use in culture. The results are expressed graphically in Fig.5. From this graph it can be seen that spleen cells from mice infected with S. typhimurium gave an enhanced response to SRBC while their response to DNP-Ficoll remained unaffected. This enhancement was maximal at 7 days post-infection and the peak of the enhancement and its magnitude were independent of the number of organisms injected. This is in direct apposition with the results of the previous experiment. However, spleen cells from animals which had received 10^3 or 10^6 organisms showed a much greater enhancement of the anti-SRBC response early in infection which was still present at higher levels 14 and 21 days after

Table 47. Effect of various doses and differing times of S. typhimurium infection on the in vitro responses to SRBC and DNP-Ficoll

Time after S. typhimurium infection (days)	No. of organisms	PFC/culture	
		Anti-SRBC	Anti-DNP
4	10	4714 ± 751	1434 ± 78
	10^3	6106 ± 202	1660 ± 44
	10^6	7806 ± 232	1486 ± 115
8	10	4106 ± 561	1686 ± 196
	10^3	3506 ± 95	1486 ± 83
	10^6	5126 ± 361	1386 ± 70
12	10	3080 ± 82	1514 ± 188
	10^3	1380 ± 60	546 ± 21
	10^6	4266 ± 164	1434 ± 90
Controls	Nil	2220 ± 95	1426 ± 84

Fig. 5. Effect of number of S. typhimurium organisms injected and the time of injection on the in vitro response to SRBC and DNP-Ficoll.

Closed triangles represent the response of spleen cells from mice infected with 10 S. typhimurium organisms.

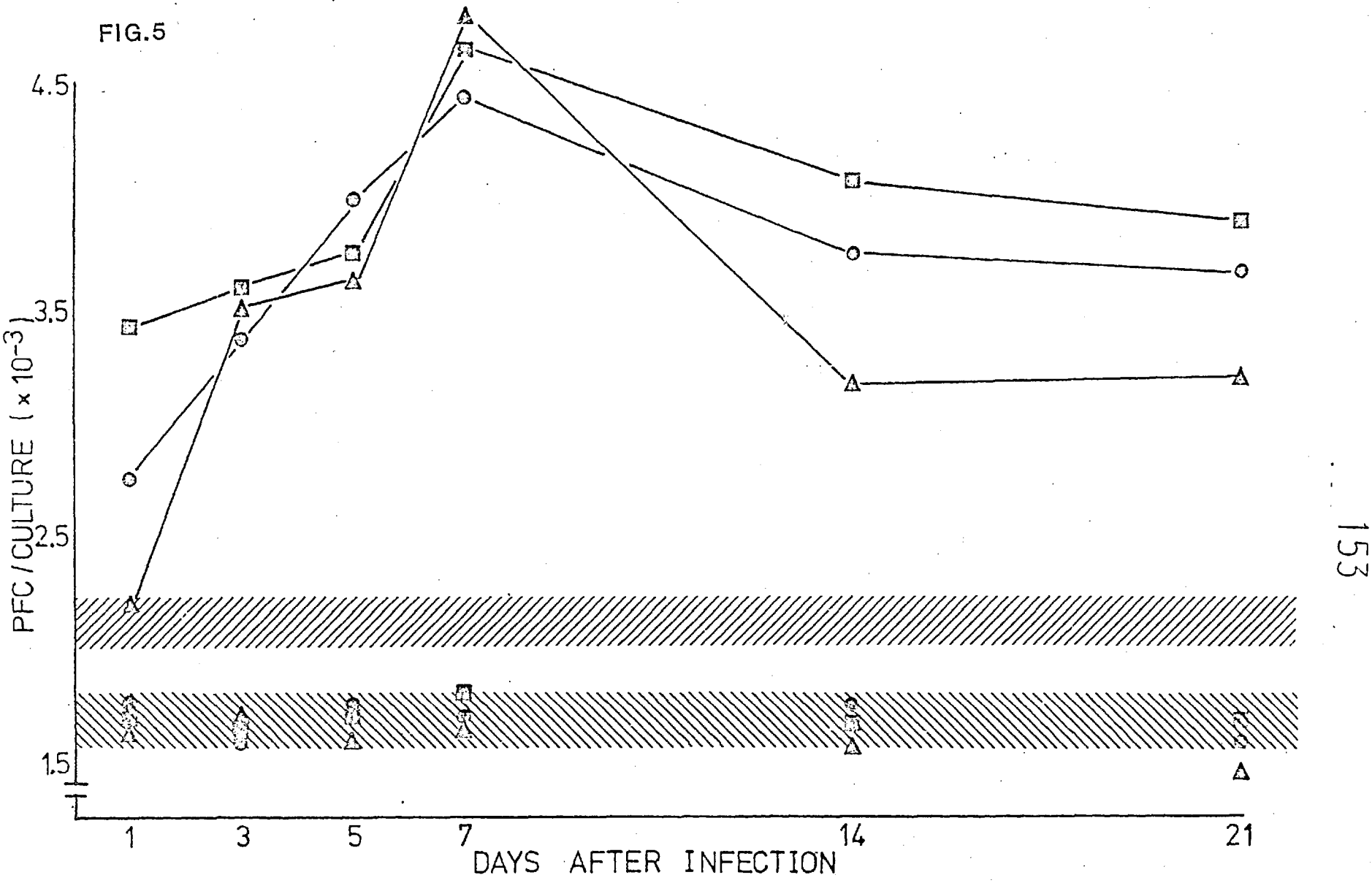
Closed circles represent the response of spleen cells from mice infected with 10^3 S. typhimurium organisms.

Closed squares represent the response of spleen cells from mice infected with 10^6 S. typhimurium organisms.

Upper cross hatching is the response of normal cells to SRBC.

Lower cross hatching is the response of normal cells to DNP-Ficoll.

FIG.5



infection. Spleen cells from mice receiving only 10 organisms showed an enhanced response to SRBC which peaked on day 7 and was of the same magnitude as that of spleen cells from animals having received 10^3 or 10^6 organisms though either side of this peak the enhancement was lower.

6:3 Malaria

Plasmodium berghei yoelii is a blood borne parasite and so its mechanism of infection is not directly comparable with those of the bacterial infections previously studied. However, there are reports indicating that the well documented suppression of the anti-SRBC response in malarial infection is due to a dysfunction of the macrophages (Loose *et al.*, 1972).

6:3:1 Time course of malarial infection and its effects on the in vitro response to SRBC and DNP-Ficoll

Mice were injected with 10^5 parasitised cells at various times ranging from 1 to 21 days before the spleens were removed for use in culture. The results are shown in Table 48 and are from 2 experiments. One experiment covered the period 1 to 4 days post-infection and the other the period 4 to 21 days post-infection. It can be seen that at 8 days post-infection the anti-SRBC response was 25% that of the normal response and at 11 and 14 days almost non-existent. By 18 to 21 days, the response was on the increase again. However, culturing spleen cells from mice 4 days after infection showed an enhancement of the response to SRBC. When the very early stages of infection were looked at spleen cells from animals from 2 to 4 days post-infection all showed an enhanced response to SRBC but the response to DNP-Ficoll was not affected. Spleen cells from day 1 of malarial infection showed a slightly depressed response to both SRBC and DNP-Ficoll; whether this was a bad set of cultures or a true result has not been determined as the cultures have not been repeated.

The response to DNP-Ficoll of cells from malaria infected animals

Table 48. Time course of the effect of malaria infection on the in vitro response to SRBC and DNP-Ficoll

Days post-malaria infection	PFC/culture			
	Anti-SRBC		Anti-DNP	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1		1660 ± 67	1220 ± 71	
2		3526 ± 183	1636 ± 76	
3		3740 ± 123	1710 ± 103	
4	3880 ± 367	3566 ± 112	1700 ± 84	
8	556 ± 128	420 ± 66		526 ± 66
11	60 ± 14	240 ± 26		306 ± 42
14	105 ± 29	470 ± 76		516 ± 54
18	900 ± 165	586 ± 57		636 ± 50
21	1136 ± 123	610 ± 13		606 ± 78
Controls	2186 ± 102	2046 ± 100	1770 ± 69	1696 ± 71

Table 49. Effect of malarial infection of mice on the antibody response of spleen cells (fractionated and recombined) in vitro to SRBC and DNP-Ficoll

Spleen cells in culture			Peritoneal cells	PFC/culture	
Unfractionated	Nonadherent	Adherent		Anti-SRBC	Anti-DNP
Normal	-	-	-	2360 ± 149	1610 ± 128
Malaria	-	-	-	900 ± 154	1300 ± 76
-	Normal	Normal	-	1756 ± 56	1500 ± 83
-	Normal	Malaria	-	270 ± 48	1286 ± 85
-	Malaria	Normal	-	1590 ± 174	1590 ± 79
-	Malaria	Malaria	-	436 ± 122	1390 ± 128
-	Normal	-	Normal	1796 ± 110	1486 ± 136
-	Normal	-	Malaria	1186 ± 93	1370 ± 151
-	Malaria	-	Normal	2030 ± 104	1450 ± 106
-	Malaria	-	Malaria	1586 ± 101	1506 ± 93
Normal + Malaria	-	-	-	500 ± 55	616 ± 102
Normal	Malaria	-	-	1960 ± 138	1610 ± 121

appears to be bi-phasic. During the period when the response to SRBC is enhanced the response to DNP-Ficoll is unaffected. However, from 8 days onwards the response was depressed in parallel with the response to SRBC and was recovering as did the response to SRBC.

6:3:2 Effect of fractionation and recombination of spleen cells from mice infected with malaria on the in vitro response to SRBC and DNP-Ficoll

Mice were infected with 10^5 parasitized cells and their spleens taken for use in culture on day 8 post-infection, when the suppression of the SRBC response was nearing its lowest point. The cells were fractionated into nonadherent and adherent cell populations and recombined with normal nonadherent and adherent cells. The results are shown in Table 49. In this experiment the response to SRBC was 40% of the normal at 8 days post-infection. When malarial adherent cells were combined with normal nonadherent cells, the response to SRBC was depressed to only just above normal background level. This depression was not as marked using peritoneal cells from infected animals to reconstitute normal nonadherent cells. Nonadherent cells from malarial infected mice responded to SRBC almost as well as normal nonadherent cells when reconstituted with normal adherent or peritoneal cells. Also, the response to DNP-Ficoll was not affected by malarial infection. One interesting aspect of this experiment was the addition of 10^6 unfractionated malarial spleen cells to normal spleen cells. This caused a depression of the SRBC response to a level even below that of infected spleen cells in culture. It also depressed the response to DNP-Ficoll. However, if this was repeated using 10^6 nonadherent malarial spleen cells then the depression was abolished.

The results obtained in this section can be summarised thus:-

1. Spleen cells from mice infected with BCG show an enhanced *in vitro* response to SRBC. The enhancement observed is dependent on the number of BCG organisms given and the time after BCG infection.

2. The response of BCG infected spleen cells to DNP-Ficoll was ambiguous.

3. Preliminary experiments suggest that macrophages mediate the enhanced *in vitro* response to SRBC.

4. Spleen cells from mice infected with *S. typhimurium* show an enhanced *in vitro* response to SRBC. The degree of enhancement observed is dependent on the number of organisms injected and the time after infection.

5. The response of spleen cells from *S. typhimurium* infected mice to DNP-Ficoll was unaffected.

6. Spleen cells from mice infected with *Plasmodium yoelii* showed a triphasic *in vitro* response to SRBC. In the first 4 days of infection, their responses were enhanced, between 8 to 14 days after infection they depressed and the response was beginning to return to normal from 18 days onwards.

7. Preliminary experiments suggested that this depression was mediated by macrophages.

8. The *in vitro* response to DNP-Ficoll was unaffected in the early stages of infection but was depressed in parallel with the SRBC response and was recovering as the SRBC response was recovering.

Acquired resistance to tuberculosis (Mackaness, 1964), brucellosis (Holland and Pickett, 1958), listeriosis (Mackaness, 1964) and salmonellosis (Collins *et al.*, 1966) is primarily cell mediated. The macrophages, which act as effectors of this type of antimicrobial immunity are mainly derived from circulating monocytes (North, 1970). It is known that when BCG is given systemically the MPS becomes hyperactive (Halpern *et al.*,

1959) and bestows a marked degree of nonspecific resistance to a variety of infectious agents (Blanden et al., 1969). It has been shown that animals infected with BCG are capable of producing enhanced levels of anti-SRBC antibody (Miller et al., 1973) and results presented here show the same effects on the in vitro SRBC response, measured in terms of PFC, of spleen cells from both BCG and S. typhimurium infected mice.

Preliminary results obtained with spleen cells from BCG infected mice suggests that this enhancement is macrophage mediated. However certain differences do arise. Firstly the response of BCG infected spleen cells to DNP-Ficoll was not consistent. In the first two experiments, there was a significant enhancement of the DNP-Ficoll response at 4, 6 and 7 weeks post-infection, whereas in the third experiment the response to DNP-Ficoll remained unaffected. This enhancement of the DNP-Ficoll response would lead one to postulate that either DNP-Ficoll is to a certain degree macrophage dependent which has been suggested by Lee et al. (1976) or that BCG infection affects lymphocyte function in some unspecified way. If the latter proposition was true, then one would expect when normal adherent cells were reconstituted with nonadherent cells from BCG infected animals that there would be an enhancement of the SRBC response. This was not the case. I should therefore like to suggest that the response to DNP-Ficoll is not affected by BCG infection and that the results showing such a phenomenon are due to some cultural abnormalities, as the response of the control cultures in one case was not as high as normally expected and furthermore all the results were very variable as can be seen from the size of the S.E. Before, however, completely negating these results the experiments should be repeated to see if my assumptions are correct.

Preliminary results obtained with spleen cells from S. typhimurium infected mice are more clearly defined that those using BCG infected spleen cells. The response to SRBC is clearly enhanced while that to DNP-Ficoll is unchanged. The interesting point about this enhancement of

the *in vitro* SRBC response is that it occurs before the bacilli become apparent in terms of viable organisms in either the spleen or the liver (Plant and Glynn, 1976). The one point of difference between the two experiments is that in the first experiment the degree of enhancement was dependent on the number of organisms injected whereas in the second experiment the peak of the enhancement was the same irrespective of the dose of organisms given, though the greater the number of organisms given the longer the enhancement remained at higher levels. Although there is no direct evidence, it can be postulated, on the basis of previous findings presented in this thesis, that this enhancement is mediated via macrophages.

The effect of *Plasmodium yoelii* infection on the *in vitro* response to SRBC produced an unpredicted result. It was found that the response to SRBC was enhanced in the very early stages of infection before the parasite could be detected in the blood. The life cycle of the parasite is such that after the animals are infected parasites cannot be found in the blood for 3 to 4 days. This is the so called pre-patent period. During this time the response to DNP-Ficoll remained unaffected. Thereafter the response to both SRBC and DNP-Ficoll was depressed. There are other reports of the depression of the *in vivo* response to SRBC (Salaman *et al.*, 1969; Greenwood *et al.*, 1971). Also the response to *E. coli* lipopolysaccharide was suppressed in chickens infected with *Plasmodium gallinaceum* (Weidanz and Rank, 1975), though this suppression was of a lesser degree than that observed to the SRBC in these infected chickens. There are also reports that malaria infection decreases resistance to infection by various microbial agents such as *S. typhimurium* (Kaye *et al.*, 1965), Moloney leukaemia virus (Wedderburn, 1970) and Toxoplasma (Strickland *et al.*, 1972). It could be postulated that this depression of both the SRBC and DNP-Ficoll responses results from a macrophage dysfunction, a T cell dysfunction or the production of suppressor T cells. Preliminary evidence presented here suggests that at least the suppression

of the SRBC response was due to a defect in macrophage function though the presence of other mechanisms can not be ruled out. The depression of the DNP-Ficoll response is more difficult to explain. It could be that all macrophages are involved in the phagocytosis of parasitized red cells. If the response to DNP-Ficoll has a requirement for adherent cells even in small numbers (Lee et al., 1976) then it is possible to imagine a situation where there are no unaffected macrophages and hence the depression of the response to DNP-Ficoll. This could also be the case in the later stages of M. lepraemurium infection when the response to DNP-POL was found to be depressed. The finding that the addition of spleen cells from Plasmodium berghei yoelii mice suppressed both the response to SRBC and DNP-Ficoll must also be remembered but it does not clarify the situation. It is possible to postulate a variety of mechanisms to explain this finding such as suppressor cells; the production of factors capable of affecting the response of normal spleen cells to both SRBC and DNP-Ficoll. Therefore, it seems that the exact mechanism of the suppression of the in vitro antibody response caused by using spleen cells from mice infected with P. yoelii awaits further analysis. It should be possible to identify the cell or cells responsible for this suppression using a system such as the Marbrook system.

CHAPTER SEVEN - EFFECTS OF CARRAGEENAN ON THE *IN VITRO* ANTIBODY RESPONSE

INTRODUCTION

Carrageenan, the main source of which is the alga Chondrus crispus, is a sulphated polysaccharide which has been fractionated into two components (Smith and Cook, 1953). One fraction, designated κ-carrageenan is present as 40% of the unfractionated extract and the other 60% is designated λ-carrageenan. For a full analysis of carrageenan's chemical structure see the work of Anderson et al. (1969). Carrageenan has been used experimentally for the induction of acute inflammatory responses but it has wide ranging effects.

Di Rosa (1972) has reviewed the biological properties of carrageenan. They can be briefly listed:-

1. Inhibition of complement activity when given intravenously due to activation of the alternate pathway;
2. Elicits hypersensitivity to cold;
3. Anticoagulant;
4. Activates Hagemann factor;
5. Induces granuloma containing large amounts of collagen.

Carrageenan is rapidly taken up by macrophages (Allison et al., 1966) and it brings about a slow release of enzymes from secondary lysosomes. The inability of macrophages to digest carrageenan has been suggested as one of the reasons that it may bring about an impairment of macrophage function and explain the peculiar feature of carrageenan granulomata in the rat which is characterized by long-lived macrophages with a slow turnover rate (Spector, 1969). It has been suggested that carrageenan is cytotoxic for macrophages (Catanzaro et al., 1971). Their electron microscope studies showed that carrageenan was taken up by macrophages stored in lysosomes which subsequently swelled and ruptured, resulting in cell death.

Carrageenan has also been shown to suppress delayed hypersensitivity in tuberculin sensitive guinea pigs (Schwartz and Leskowitz, 1968). Bice et al. (1971) found that not only did carrageenan suppress the expression

of an existing delayed hypersensitivity reaction to BSA but when administered at the time of immunization, it suppressed the induction of delayed hypersensitivity in guinea pigs. This suppression of delayed hypersensitivity has also been shown in mice (Boros and Shwartz, 1975). λ -carrageenan has been shown to depress the humoral antibody response in vivo to SRBC (Aschheim and Raffel, 1972). This effect appeared to be unrelated to an induced malfunction of macrophages. This they decided on finding that on injecting SRBC into the peritoneum of normal and carrageenan treated animals and finding that both experimental and control groups took up SRBC to the same extent as assessed by microscopical means. They concluded that cells other than macrophages were affected. The possibility that carrageenan mediates its suppressive effects by serum factors was raised in the work of Schwartz and Catanzaro (1973). They were trying to generate delayed type hypersensitivity lesions of equal intensity using antigen, skin reactive factor (SRF) and Con A. Carrageenan suppressed the antigen induced delayed hypersensitivity reactions to a greater extent than those reactions induced by SRF or Con A. They concluded that if carrageenan suppressed merely by an effect on macrophages the suppression of all three types of lesion should have been equivalent. As they found that they were not, they postulated the existence of serum factors.

Carrageenan has also been used in studies on the induction of tolerance (Lukic and Leskowitz, 1974; Lukic et al., 1975). Susceptibility to the induction of tolerance with ultracentrifuged bovine gamma globulin is dependent on the mouse strain used. Pretreatment of mice resistant to tolerance induction with carrageenan leads to a loss of their resistance to tolerance, while treatment with cobra venom factor had no effect. Stimulation of tolerance susceptible animals with BCG leads to a reduction of their susceptibility to tolerance induction. On the basis that carrageenan is cytotoxic for macrophages and BCG is known to stimulate the MPS, they suggested that these results provide evidence for the concept that the differential susceptibility to the induction of tolerance

is related to macrophage function.

On the basis that carrageenan may affect macrophage function it was decided to ascertain the effect of carrageenan pretreatment on the *in vitro* antibody response. Also, the combined effects of *C. parvum* and carrageenan on the *in vitro* antibody response were studied.

RESULTS

The work described in this section was done in collaboration with Vivian Rumjanek who was interested in the effect of carrageenan pretreatment on the cell mediated arm of the immune response, with special reference to the inflammatory response. Therefore for a wider discussion of the effects of carrageenan reference should be made to her thesis (1976). This work arose from reports in the literature that carrageenan was cytotoxic for macrophages *in vitro* (Catanzaro *et al.*, 1971) and also that carrageenan pretreatment led to a suppression of the *in vivo* humoral response to SRBC. Therefore, the effect of carrageenan pretreatment on the *in vitro* response to both SRBC and DNP-Ficoll was studied and also the effect of combining carrageenan and *C. parvum* pretreatment on the *in vitro* response was observed.

7:1 The effect of carrageenan pretreatment on the in vitro response to SRBC and DNP-POL or DNP-Ficoll

Spleens from mice which had received either 1 injection 24 hours before or 4 injections every other day for a week prior to use, of carrageenan, were removed and cultured *in vitro* to assay their response to SRBC and DNP-POL or DNP-Ficoll. The results are shown in Table 50, which is a summary of 2 such experiments. It can be seen that carrageenan pretreatment depressed the response to SRBC. Maximal suppression was achieved by pretreating the mice with 4 doses of carrageenan. The response to DNP-POL or DNP-Ficoll was unaffected whether the mice were given 1 dose or several doses of carrageenan. When cell fractionation and recombination experiments were performed it can be seen that both adherent spleen cells and peritoneal cells from carrageenan animals were defective in their ability to reconstitute the *in vitro* antibody response of normal nonadherent spleen cells. The defect was most marked when the animals had been given 4 doses of carrageenan. However, it was possible to reconstitute nonadherent cells from carrageenan animals with normal peritoneal cells.

Table 50. Effect of carrageenan pretreatment on the in vitro response to SRBC and DNP-POL of whole spleen cell and fractionated populations

Spleen cells in culture			Peritoneal cells	PFC/culture			
				Anti-SRBC		Anti-DNP	
Unfractionated	Nonadherent	Adherent		Exp. 1	Exp. 2	Exp. 1	Exp. 2
Normal				2210 ± 93	2190 ± 99	1606 ± 133	1700 ± 78
C-1				1016 ± 98	1376 ± 111	ND	1610 ± 31
CX				296 ± 72	666 ± 79	1420 ± 59	1686 ± 81
	Normal	Normal		1700 ± 50	1770 ± 60		
	Normal	C-1		1360 ± 88	990 ± 99		
	Normal	CX		716 ± 156	516 ± 54		
	Normal		Normal	1610 ± 92	1776 ± 88		
	Normal		C-1	1110 ± 223	1196 ± 94		
	Normal		CX	686 ± 161	496 ± 75		
	Normal		Normal	1586 ± 57			
	C-1		Normal	1536 ± 83			
	CX		Normal	1566 ± 108			
	Normal				206 ± 26		1470 ± 99
	C-1				256 ± 62		ND
	CX				166 ± 33		1566 ± 128

Cell types and numbers:- Normal = untreated; C-1 = given 1 dose of carrageenan 24 hours previously; CX = 4 do of carrageenan every other day for 1 week before use in culture. Unfractionated - 20 x 10^6 cells/culture; nonadherent - 13 x 10^6 cells/culture; adherent - 10^6 cells/culture and peritoneal cells - 10^6 cells/culture.

7:2 Effect of carrageenan and C. parvum pretreatment on the in vitro response to SRBC

Mice were pretreated in a variety of ways for this experiment. They can be designated thus:-

1. Normal = untreated mice

2. C-1 = mice given 1 injection of carrageenan 24 hours prior to the removal of their spleens

3. C-7 = mice given 1 injection of carrageenan 7 days prior to removing their spleens

4. CX = mice given 4 doses of carrageenan every other day for a week prior to removal of their spleens

5. C.p.-5 = mice given C. parvum 5 days before the removal of their spleens

Mice received various combinations of these treatments and their spleens removed and used in culture. The results are shown in Table 51. The response to SRBC was depressed by pretreatment of the animals with carrageenan as previously found and C. parvum pretreatment caused its usual enhancement of the response. However, if spleen cells from C-7: C.p.-5 mice were cultured the in vitro response was depressed to levels found in CX mice. This is to be compared with culturing C-1: C.p.-5 cells which gave a restored response to SRBC. This restoration was not as great as the enhancement from cells only pretreated with C. parvum but it was enhanced in comparison to C-1 cells. Cell fractionation and reconstitution experiments were also carried out. When normal nonadherent spleen cells were reconstituted with adherent spleen cells from the various groups, it was found that C.p.-5 adherent cells restored and enhanced the response of normal nonadherent cells as previously observed. C-1 and C-7 adherent cells depressed the response to varying degrees. The interesting response was that of the C-7:C.p.-5 adherent cells when compared to the C-1:C.p.-5 cells. The former suppressed the response while the latter enhanced it. The same pattern of responses was found when peritoneal cells were used

Table 51. Effect of carrageenan and C. parvum pretreatments on the in vitro response to SRBC of whole spleen cell populations and fractionated cell populations

Cell types and numbers:-

Normal = untreated mice;
C-1 = mice having received 1 dose of carrageenan 24 hours previously;
C-7 = mice having received 1 dose of carrageenan 7 days previously;
CX = mice having received 4 doses of carrageenan every other day for a week prior to use as spleen cell donors;
C.p.-5 = mice having received C. parvum 5 days earlier.

Unfractionated - 20×10^6 cells/culture
Nonadherent - 15×10^6 cells/culture
Adherent - 10^6 cells/culture
Peritoneal cells - 10^6 cells/culture.

Table 51. Effect of carrageenan and C. parvum pretreatments on the in vitro response to SRBC of whole spleen cell populations and fractionated cell populations

Spleen cells in culture			Peritoneal	PFC/culture
Unfractionated	Nonadherent	Adherent	cells	Anti-SRBC
Normal	-	-	-	2190 ± 198
C-1	-	-	-	1380 ± 129
C.p.-5	-	-	-	4640 ± 305
C-7:C.p.-5	-	-	-	290 ± 88
C-1:C.p.-5	-	-	-	2816 ± 160
CX	-	-	-	490 ± 91
-	Normal	Normal	-	1760 ± 165
-	Normal	C-1	-	1416 ± 120
-	Normal	C.p.-5	-	3586 ± 204
-	Normal	C-7:C.p.-5	-	276 ± 53
-	Normal	C-1:C.p.-5	-	3186 ± 172
-	Normal	C-7	-	396 ± 69
-	Normal	-	Normal	1740 ± 80
-	Normal	-	C-1	1216 ± 102
-	Normal	-	C.p.-5	3630 ± 225
-	Normal	-	C-7:C.p.-5	860 ± 147
-	Normal	-	C-1:C.p.-5	3310 ± 191
-	Normal	-	C-7	296 ± 65
Normal	-	-	Normal	2500 ± 195
CX	-	-	Normal	1516 ± 145

to reconstitute normal nonadherent cells.

Therefore, it can be concluded that if carrageenan was given before C. parvum it was possible to negate the enhancing effect of C. parvum. Whereas if C. parvum was given first, an enhancement of the in vitro response was obtained. As a footnote to this experiment it was found that the addition of 10^6 normal peritoneal to CX spleen cells was capable of restoring the response of these cells to a great extent. Though the restoration led to a response that was not as good as the response acheived by normal spleen cells, it was significantly better than CX spleen cells alone.

DISCUSSION

The results of this section can be summarised thus:-

1. Pretreatment with carrageenan depressed the *in vitro* SRBC antibody response.

2. This depression was more marked in cells from animals that had received 4 doses of carrageenan every other day for a week or 1 dose 7 days previously, before the removal of their spleens for use in culture. The depression was still significant if only one dose of carrageenan was given 24 hours before the removal of spleens.

3. The responses to both DNP-POL and DNP-Ficoll were not affected.

4. The depression of the SRBC response was mediated by macrophages.

5. Spleen cells from animals given carrageenan before *C. parvum* pretreatment no longer showed the characteristic enhancement of the *in vitro* anti-SRBC response.

6. Spleen cells from animals given carrageenan after *C. parvum* pretreatment showed either a restored or an enhanced response to SRBC.

One of the first points to be noted in this study of the effect of carrageenan on the immune response was its effects on macrophages, *per se*, from both the peritoneal cavity and from the spleen. If, as has been reported, carrageenan is cytotoxic for macrophages (Catanzaro *et al.*, 1971) one might expect to find a decreased number of macrophages from both sites. This, however, was not the case and in fact peritoneal cells from carrageenan treated animals consistently gave higher yields than the controls. These cells were viable as assessed by the fluorescence method and were also larger than normal peritoneal cells. It is, of course, possible that carrageenan is cytotoxic for macrophages and as a concomitant of cell death there is a proliferation of macrophage precursors in the bone marrow which repopulate the depleted areas. With respect to this argument, work done by Rumjanek and Šljivić (personal communication) on the uptake of ^{51}Cr-SRBC appears relevant. They found that the uptake of labelled SRBC was depressed after 1 dose of carrageenan but normal after

4 doses. This would appear to suggest that carrageenan has an initial suppressive effect on macrophage function which is overcome with time.

Before discussing these results in detail, others, obtained by Ms. Rumjanek, will be elaborated in an attempt to make any explanation seem clearer. (For further details of experimental methods and a fuller exposition of the results see Ms. Rumjanek's thesis (1976).) Looking at the response of spleen cells from carrageenan animals to PHA, she found that if animals had been given carrageenan every other day for a week i.e. 4 doses of carrageenan or 1 dose 7 days before, there was a suppression of the PHA response. If, however, animals had received only 1 dose 24 hours before, the response to PHA was variable i.e. in two experiments out of 4 the response was the same as the controls and in the other two experiments it was enhanced. The response to LPS was variable, either it was the same as the controls or else it mirrored the PHA response. Using mesenteric lymph node cells, 4 doses of carrageenan led to a depression of the PHA response whereas 1 dose given 7 days beforehand had no effect. The lack of effect of carrageenan pretreatment 7 days previously could be due to the route of injection, as carrageenan was always given intraperitoneally. One dose 7 days previously might be enough to affect the spleen but possibly not enough would reach the lymph nodes and so only after 4 doses was there sufficient carrageenan in the nodes to cause an observable effect. Lymph node cells from animals given carrageenan 24 hours previously showed either a slightly enhanced or normal response to PHA. On removing the adherent cells from all the groups of spleen cells, the PHA response though lower was the same in all groups. The addition of peritoneal cells, from mice given 4 doses of carrageenan, to normal nonadherent spleen cells caused a depression of the PHA response in comparison with the addition of normal peritoneal cells or peritoneal cells from animals given 1 dose of carrageenan 24 hours previously.

From the results of the <u>in vitro</u> antibody response, it can be seen that macrophages from carrageenan treated animals mediated a suppression

of the in vitro response to SRBC. Spleen cells from animals pretreated with carrageenan and subsequently given C. parvum did not show an enhanced response to SRBC. They were, in fact, very suppressed. In contrast, cells from mice given C. parvum before carrageenan gave a restored though not significantly enhanced response to SRBC. Macrophages from C-7:C.p.-5 mice depressed the response to SRBC of normal nonadherent cells, whereas macrophages from C-1:C.p.-5 mice enhanced the response to SRBC.

These two sets of data are very difficult to interpret when trying to produce a coherent model for the action of carrageenan. It can be seen that a difference exists between the effect of 1 dose and 4 doses of carrageenan. The latter suppresses both the in vitro response to SRBC and PHA while not affecting the uptake of chromium-labelled SRBC. The former has no effect on the PHA response but depresses both SRBC uptake and the anti-SRBC response in vitro. While carrageenan given as 4 doses every other day for a week or 1 dose 7 days prior to the removal of spleens affects both the macrophages and T cells, it must be postulated that the effect on T cells is mediated via macrophages. The possible mechanisms are open to conjecture but it could be that the macrophages exert either a direct cell-to-cell suppressive effect or that there is the production of a suppressive factor(s). The initial depression of the in vitro SRBC response may result from a lowering of the number of macrophages present in the animals and/or altered handling and presentation of antigen by macrophages as reported by Wiener and Bandieri (1974) in the case of Biozzi high and low responders. The results combining C. parvum and carrageenan treatment show that carrageenan given before C. parvum prevents the development of the normally enhanced response to SRBC brought about by C. parvum on its own. It is known from work earlier in this thesis that for the enhancement of the SRBC response, due to C. parvum pretreatment, is mediated by macrophages and that T cells are needed for the developement of such macrophages. However, this still does not allow elucidation of the mechanism by which carrageenan suppresses the

antibody and PHA responses. All that can be said is that in the two days between giving carrageenan and C. parvum some mechanism is activated which prevents C. parvum from exerting its normal adjuvant effect. It therefore needs more experiments to assess the mechanism of action of carrageenan. This should be possible using a system such as the Marbrook system.

CHAPTER EIGHT - A T CELL FACTOR : ITS GENERATION AND ASSAY

INTRODUCTION

There is now ample evidence that the production of antibodies by B cells *in vivo* is greatly enhanced by the presence of T cells (Claman *et al.*, 1966). This phenomenon is referred to as T-B cell cooperation and it is the origin of the concept that many antigens are thymus dependent. This phenomenon was first studied *in vivo* but there is now ample evidence for the same thing occurring *in vitro*. Recent work has shown that the mechanism of T-B cooperation *in vitro* involves several different types of signals, some of which are mediated by soluble factors derived from T cells. This area is somewhat confusing as there appears to be a multiplicity of factors and competing hypotheses. To simplify this it can be postulated that B cell activation *in vitro* needs two signals (Watson *et al.*, 1973). The first one is generated by the interaction of antigen with specific antigen receptors on the surface of the B cell; the second signal appears to be a soluble factor derived from T cells. However, there are several different ways of generating this T cell factor.

1. T cell factors - enhancing the immune response

A. Nonspecific factors

There is plentiful evidence that when T cells are activated they produce a factor which will act as a second signal for B cell activation. This factor is nonspecific. The activation of T cells can be either activated by antigens or alloantigens.

T cell activation *in vivo* has been studied by activation using allogeneic antigens. This *in vivo* allogeneic effect has been reviewed by Katz and Benacerraf (1972). More recently, it has been shown that allogeneic cells or supernatants from T cells activated by allogeneic antigens could replace the need for T cells in T cell depleted cell populations *in vitro*. Dutton and his co-workers (Hirst and Dutton, 1970; Dutton *et al.*, 1971) were the first to observe that allogeneic T cell supernatants could reconstitute the antibody response of T cell depleted populations *in vitro*. Doria *et al.* (1972) confirmed this finding. Schimpl and Wecker (1971;

1973) were able to restore both the in vitro IgM and IgG responses of T cell depleted populations to SRBC with a factor derived from spleen cells allogeneically stimulated. Stimulation with specific antigens has also been shown to release this T cell replacing factor (TRF) (Geha et al., 1973; Gorczynski et al., 1972; 1973a,b; Rubin and Coons, 1972a,b). A similar factor has been found in the supernatants of lymphoid cell lines (Rubin et al., 1974). These factors all have similar properties - they are produced by T cells, nondialyzable heat stable, resistant to DNase and RNase but inactivated by pronase.

The B cell activation by T cell mitogens (Elfenbein et al., 1973; Geha et al., 1974; Piguet and Vassali, 1972) is suggested to be mediated through TRF. Andersson et al. (1972a,b) found that B cells from thymectomized, irradiated, bone marrow reconstituted mice treated with anti-theta and complement failed to respond to soluble Con A. The response was restored if supernatants from Con A stimulated T cell cultures were added.

Katz and his coworkers have reported an allogeneic effect factor (AEF) (Armerding et al., 1975). In their system thymocytes of one strain were activated in vivo against F_1 hybrid cells for 7 days. At the end of this time the spleens were removed and co-cultured with the same F_1 hybrid cells (irradiated) in vitro for 24 hours. Then the supernatants were assayed for their ability to support an in vitro response to SRBC. Unabsorbed AEF was able to reconstitute the response of T cell depleted spleen cells in vitro. This ability could be absorbed by antisera against mouse β_2 microglobulin leading them to suggest that AEF possesses antigenic determinants identical to or cross reacting with β_2 microglobulin.

Cerny et al. (1975) reported an immunoenhancing factor (IEF). In their system, spleen cells from mice immunized with tetanus toxoid 60 days previously were cultured with tetanus toxoid for 48 hours and the supernatant activity assayed in vitro. It was found to non-specifically

augment the antibody response of T depleted spleen cells to antigenic stimuli and was characterized as heat stable and a protein with a molecular weight of around 75,000 daltons.

B. Specific T cell factors

Feldmann and Basten (1972) have produced evidence that besides a non-specific T cell factor, which was dialyzable in opposition to the findings of Rubin and Coons (1972) and Gorczynski et al. (1972), there is a specific T cell factor active in their in vitro system. This specific factor is a nondialyzable, antigen specific mediator made by syngeneic T cells. This factor unlike TRF requires the presence of macrophages and does not act directly on B cells. Feldmann characterized it to be a monomeric IgM that is cytophilic for macrophages (1972). They proposed that antigen specific monomeric IgM complexed with antigen and this complex bound to macrophages. The B cell interacts with this complex on the macrophage surface and is thereby activated. The specific factor of Feldmann is to be contrasted with that of Taussig (1974). He reported a factor with in vivo activity which was produced by activating T cells to antigen in irradiated animals and then culturing these activated T cells in vitro in the presence of the same antigen to obtain the supernatant. This was then transferred with bone marrow cells and antigen into lethally irradiated recipients and their response to the antigen assayed. He and his co-workers showed that the factor had a binding site for antigen, that it was not an immunoglobulin and that its molecular weight was 50,000 daltons. It did however react with anti H-2 sera. These properties suggested to them that their T cell factor was a soluble expression of the T cell antigen receptor. Further studies suggested that it was coded at least in part by the I region of the H-2 locus (Munro et al., 1974; Munro and Taussig, 1975).

Tada and his co-workers have isolated a soluble enhancing factor from the thymocytes or spleen cells of rats hyperimmunized with DNP-Ascaris. This factor when injected into neonatally thymectomized rats

was found to restore their ability to produce IgE antibody against DNP-Ascaris. This factor was specific in its activity and on characterization it was found to be of molecular weight 100,000-200,000 daltons and was found to have Fab and μ determinants (Taniguchi and Tada, 1974). This makes it very similar to the specific factor described by Feldmann.

2. T cell factors suppressing the immune response

Feldmann (1974a,b) has also reported two factors produced by activated T cells that are capable of suppressing the in vitro immune response. There was both a specific and a nonspecific suppressive factor. Chemical characterization of the specific factor showed that its activity could be removed with polyvalent anti-mouse immunoglobulin, anti-κ and anti-μ chain antibody. However whether the apparent resemblance of the specific enhancing and suppressive factors meant that they were the same factor awaits further analysis.

Rich and Pierce (1974) reported that mouse spleen cells activated by Con A secreted a factor(s) which is a potent, non-cytotoxic inhibitor of the primary immune response to SRBC in vitro. This factor suppressed the background, IgM and IgG responses. Though its precise mode of action was not known they suggested that it appeared to act during an early antigen-dependent stage of the in vitro response to abort full expression of the PFC response during the later exponential expansion of plaque forming clones.

Tada and his co-workers have also demonstrated an antigen specific T cell component that suppressed the IgE response in the rat (Tada et al., 1973; Okumura and Tada, 1974). This factor, as the enhancing factor, was extractable from mechanically disrupted thymocytes or spleen cells from DNP-Ascaris primed animals. The suppressor molecule was however chemically different from the enhancing factor in that it had no immunoglobulin determinants and was less than 100,000 daltons molecular weight. Its activity could be absorbed with heterologous anti-thymocyte serum. They also found a similar antigen specific suppression by T cells primed

to KLH of the IgM and IgG responses of Balb/c mice primed to DNP-KLH (Tada and Takemori, 1974; Takemori and Tada, 1974). They found that this T cell suppression was mediated by a factor which was specific for the carrier molecule to which the animals had been primed and its activity was absorbed with antisera reactive to surface determinants of T cells and anti H-2 antibodies. Thus, it is analogous to the factor reported by Taussig and Munro though their factor enhances rather than suppresses.

Finally, Zembala et al. (1975) reported their findings concerning the characterization of a factor which was capable of depressing the passive transfer of contact sensitivity to picryl chloride by immune cells. This factor appeared to be the only one reported that is capable of depressing a cell mediated immune response in vivo. It was antigen specific, had a molecular weight of approximately 50,000 and could be absorbed by specific antigen in the form of picrylated albumin attached to Sepharose beads.

Therefore, it was attempted to produce a factor in vitro from primed spleen cells which could be assayed in vitro. Initially experiments were performed in double Marbrook vessels and from then on primed spleen cells were cultured in vitro to give rise to this factor.

RESULTS

8:1 The effect of culturing cells from KLH primed mice with normal cells in double Marbrook vessels

Spleen cells from mice previously primed to KLH were cultured in the inner chamber of Marbrook vessels (double) at a final cell number of 3 x 10^6 KLH primed cells/vessel with 0.15 µg DNP-KLH. Normal cells were cultured in the outer vessel at a final cell number of 30 x 10^6 cells/vessel in the presence of 1.5 µg DNP-KLH. The two cell types were separated by a cell impermeable membrane and the response to DNP-KLH was assayed 4 days later. The results are shown in Table 52. It can be seen that unprimed cells did not respond to DNP-KLH, however if the cells were primed with KLH prior to use in culture they gave a response to DNP-KLH. It was possible to induce a response in unprimed cells by culturing them in the presence of cells previously primed to KLH in double Marbrook vessels, even though they were separated by a cell impermeable membrane. The response thus induced was comparable to that of primed cells. On the basis of this experiment, it was postulated that the primed cells produced a factor or factors capable of potentiating the response of unprimed spleen cells. Further experiments were carried out to test this possibility.

8:2 The production and assay of a factor(s) from KLH primed spleen cells

In an attempt to produce a factor from KLH primed spleen cells which had a biological activity, single spleen cell suspensions from both KLH primed and untreated mice were prepared and cultured in Petri dishes at a concentration of 10 x 10^6 cell/ml for 1 hour. At the end of this time, the cells were separated into adherent and nonadherent cell populations. The nonadherent cells were at a concentration of 7.5 x 10^6 cells/ml and the adherent cells were those remaining on the Petri dish after it had been thoroughly washed though their concentration was not known. These cell populations plus unfractionated spleen cells at a concentration of 10 x 10^6 cells/ml were cultured in Petri dishes for 24 hours in medium

Table 52. Effect of culturing KLH primed spleen cells and normal spleen cells and normal spleen cells separated by a cell impermeable membrane on the antibody response to the DNP-KLH in vitro of the unprimed cells

Contents of Inner Vessel	Contents of Outer Vessel	PFC/culture ANTI-DNP
KLH primed spleen cells + DNP-KLH	Normal spleen cells + DNP-KLH	1240 ± 235
Nil	Normal spleen cells + DNP-KLH	50 ± 100
Nil	KLH primed spleen cells + DNP-KLH	1770 ± 111

Cell number and antigen doses:-

Inner chamber - 3×10^6 KLH primed cells : 0.15 µg DNP-KLH.

Outer chamber - 30×10^6 normal or KLH primed cells : 1.5 µg DNP-KLH.

containing 1 μg DNP-KLH/ml culture medium. Then the supernatants were removed and spun at 2000 rpm for 5 minutes. 1 ml of the supernatants thus derived was added to 20 x 10^6 unprimed spleen cells which were cultured for 4 days in single Marbrooks in the presence of 1 μg/ml DNP-KLH. At the end of the culture period the response of the unprimed cells to DNP-KLH was assayed. The results are shown in Table 53.

It can be seen that supernatants from unfractionated and nonadherent KLH primed spleen cells potentiated the response of unprimed cells to DNP-KLH and that this potentiation was optimal if a 1/10 dilution of the supernatant from unfractionated cells was used. Supernatants from either unfractionated or fractionated cell populations of unprimed cells caused no potentiation of the response of normal spleen cells. Supernatants from cell fractions of KLH primed cells still caused some effect on unprimed cells. Therefore, it was possible to produce a factor from primed cells in vitro and to assay its activity in vitro.

8:3 The effects of various doses of supernatant from KLH primed cells on the in vitro response of unprimed cells to DNP-KLH

Dilutions of the supernatant from cultures of unfractionated KLH primed spleen cells were cultured with normal spleen cells and 1 μg DNP-KLH and at the end of this time the response to DNP-KLH assayed. The results are shown in Table 54. Dilutions of 1/10 or 1/20 led to the best potentiation of the response while dilutions of 1/80 or lower ceased to have a potentiating effect on the response.

8:4 The production and assay of a factor from purified T cells from KLH primed spleen cells

Unfractionated spleen cells and nylon wool purified T cells from KLH primed animals were cultured as described in 8:2 and the supernatants from these cultures tested for their ability to potentiate the response to DNP-KLH of unprimed spleen cells. The results of this experiment are shown in Table 55. It can be seen that supernatants from T cells were capable of potentiating the response of unprimed cells to DNP-KLH and

Table 53: Effect of the addition of supernatants from primed and unprimed spleen cell populations on the in vitro response of unprimed spleen cells to DNP-KLH

Origin of the supernatant added to cultures	PFC/culture Anti-DNP response
Normal unfractionated spleen cells	32 ± 15
Normal nonadherent spleen cells	0
Normal adherent spleen cells	0
Normal unfractionated spleen cells (1/10)	0
KLH primed unfractionated spleen cells	240 ± 20
KLH primed nonadherent spleen cells	97 ± 40
KLH primed adherent spleen cells	17 ± 6
KLH primed unfractionated spleen cells (1/10)	534 ± 55

1/10 = 1/10 dilution of supernatant from unfractionated normal and primed spleen cells.

Table 54. Dose response of supernatant from KLH primed cells on the in vitro response to DNP-KLH

Dilution of supernatant from KLH primed spleen cells	PFC/culture Anti-DNP response
Neat	370 ± 54
1:10	480 ± 61
1:20	400 ± 54
1:40	296 ± 44
1:80	16 ± 10
1:160	40 ± 18
Nil	45 ± 17

Table 55. Effect of the addition of supernatants from unfractionated KLH primed spleen cells and purified T cells from KLH primed spleen cells on the in vitro response of unprimed spleen cells to DNP-KLH

Origin and dilution of supernatant added to cultures	PFC/culture Anti-DNP response
Unfractionated KLH primed cells (1:10)	466 ± 86
" " " " (1:20)	690 ± 60
" " " " (1:40)	346 ± 103
Purified T cells from KLH primed spleens (1:10)	206 ± 62
" " " " " " (1:20)	836 ± 50
" " " " " " (1:40)	460 ± 88
Nil	100 ± 45

that a 1/20 dilution of the supernatant gave the best effect. Supernatants from unfractionated primed spleen cells were again found to have a potentiating effect on the response of unprimed cells. This effect was also better when a 1/20 dilution of the supernatant was used.

DISCUSSION

The results in this section can be listed thus:-

1. Using double Marbrook vessels, spleen cells from animals primed to KLH could induce an in vitro response to DNP-KLH in unprimed spleen cells when separated by a cell impermeable membrane.

2. Supernatants from spleen cells primed to KLH and cultured in the presence of DNP-KLH for 24 hours were capable of inducing a response to DNP-KLH in unprimed spleen cells in culture.

3. Supernatants from T cells primed to KLH were able to potentiate a response to DNP-KLH in unprimed cells.

Feldmann and Basten (1972) were the first to report the generation of a specific helper factor by T cells activated to KLH which was capable of inducing a response to DNP-KLH in B cells separated from the T cell source by a nucleopore membrane. Results presented in this section confirm that this type of experiment is possible. The exact reconstruction of the work has not been attempted but double Marbrooks have been used to show the presence of a factor produced by primed spleen cells. The results reported here give no indication as to the specificity of this factor. However, preliminary results from experiments still in progress suggest that the activity of this factor can be removed by absorption with both KLH and anti-mouse globulin. These tentative findings if proven would confirm the findings of Feldmann (1972) that there is a specific helper factor which is immunoglobulin in nature.

The activity of the supernatant was found to be optimal at a 1:20 dilution. This suggests that whatever the factor's mode of action when it is added to the cultures in an undiluted form it caused the cells to give a lower response to DNP-KLH. This was not due to any cytotoxic effect as all the cultures had the same viability. This is similar to other findings which suggest that it is possible to have too much T cell help (Feldmann, 1975) present in the culture medium. This factor could also be produced by nylon wool purified T cells. This method, as

previously stated, gives a population of cells which are 95-97% pure T cells. Whether this factor is produced by T cells or is just due to the release of material from the surface of the cells which they have previously acquired *in vivo* cannot be clearly assessed even by this method. Ways to prove that this factor is synthesised wholly by T cells would be (1) treat a population of pure T cells with trypsin and then to culture them and see if the supernatants of such cells were active *in vitro*; (2) use inhibitors of protein synthesis to see if the factor was still produced, and (3) try to extract the factor from dead cells.

One of the interesting things to have done during the production of this factor was to have monitored the production of this factor on a time basis i.e. to harvest the supernatants every 24 hours and to assay their activity *in vitro*. It is possible that such a factor is produced initially by the T cells after antigenic stimulation in the first 24 hours. Activated lymphocytes, irrespective of whether they proceed through mitosis or not, secrete a number of biologically active proteins often a short time after antigenic stimulation (Wheelock, 1965; Williams and Granger, 1969; Bach *et al.*, 1971; Pick *et al.*, 1970) and so it is possible that these primed spleen cells would only produce this factor for a short period of time while in culture.

Another of the things not checked in this brief report was whether the factor needed to operate in a syngeneic system or whether it was capable of acting across histocompatibility barriers.

This section is a brief report of the production of a T cell factor with *in vitro* activity. Its existence will be used to give weight to the argument to be postulated in the general discussion.

CHAPTER NINE - GENERAL DISCUSSION

In this section a possible model of macrophage activation, as observed in this thesis, will be postulated. This would serve to explain some of the findings and act as a working basis for planning further experiments. As most of the work done in this thesis was using C. parvum this will be taken as the main source of information in the construction of this model.

When mice are pretreated with C. parvum or infected with BCG, M. lepraemurium or S. typhimurium, it is possible that these agents affect macrophages per se or they act via the mediation of T cells. Though the first mechanism is the simpler of the two, no evidence for its existence could be found in the work done in this thesis. Macrophages could only be activated, with respect to their in vitro performance in antibody response to SRBC, via the auspices of T cells.

This pathway of macrophage activation is similar to the system Mackaness proposes for the production of activated macrophages during the course of some infections. On injection of C. parvum or infection of the mice, bacilli are taken up by the macrophages and the presence of them in these cells causes a triggering of the T cells. Therefore, T cells as well as macrophages can be said to be primed by the presence of these agents. Whether the interaction between macrophages and T cells is by cell to cell contact or by means of a factor(s) cannot be said. However, it is known that macrophages are needed for the generation of helper T cells (Erb and Feldmann, 1975a,b) and that this help is mediated by soluble factors. Both pathways may, therefore, be possible. It is also a possibility that T cells can be primed without the intervention of macrophages, so one can postulate two pathways for the involvement of T cells in the activation of macrophages. Once T cells have responded to these agents by direct or indirect means, they secrete a factor(s) which acts on macrophages making them capable of more efficient interaction with B cells. In the case of macrophages activated in response to bacterial infection, as in the work of Mackaness (1971), it causes them to

become more efficient in bacterial killing. Whether it is the same population of macrophages which perform both functions or whether two or more functionally distinct macrophage subpopulations exist can only be speculated upon.

Evidence for the production of a T cell factor capable of activating macrophages can be found in the work of David and others (reviewed by David, 1975) and work done in this thesis would indicate that supernatants from T cells primed to KLH potentiate an in vitro response to DNP-KLH in unprimed cells. Furthermore, there appears to be evidence, again from this thesis, for the production of a factor by C. parvum primed spleen cells, when cultured with SRBC and C. parvum, that enhances the antibody response to both SRBC and DNP-KLH. This effect of T cells on macrophages must only be of short duration as nonadherent cells from C. parvum pretreated animals reconstituted with normal adherent cells never gave a response above that of normal spleen cells. However, this could be altered if C. parvum was added in vitro to the cultures, indicating that previously sensitized lymphocytes had to be restimulated before they could effect any change on the macrophage population. Once activated macrophages appear to be so for long periods of time. Spleen cells 21 days after pretreatment with C. parvum still showed an enhanced response to SRBC and macrophages from these animals maintained in vitro for 5 days retained some of their activity. This activation can be presumed to allow the macrophages to be more efficient in antigen presentation and other roles involved in the triggering of B cells, hence the enhanced response to SRBC.

With regard to the temporal relationship between T cell stimulation of macrophages (i.e. that though spleen cells from animals pretreated with C. parvum 5 days previously showed an enhanced response to SRBC, the nonadherent cells from these animals did not affect the response of normal macrophages), it would be of interest to take spleen cells from animals pretreated at various times between 1 and 5 days to see if it can

be shown at any time during this period that the nonadherent cells could enhance the *in vitro* SRBC response. Another experiment to perform would be to take macrophages from *C. parvum* treated animals and see if when added to TXB mice it is possible to bypass the need for T cells in the response to SRBC using activated macrophages.

With respect to the effect of infection on the *in vitro* response of spleen cells to SRBC, an enhancement was observed when mice had been infected with BCG, *M. lepraemurium*, *S. typhimurium* and in the early stages of malaria infection. As previously stated, whether this activation is mediated by the same macrophages as are capable of enhanced bacterial killing is open to conjecture. One point of interest is the time at which these enhanced responses can be detected. In the cases of BCG and *M. lepraemurium*, it is 4 to 6 weeks after infection of the animals but in the cases of *S. typhimurium* and *Plasmodium yoelii*, it is 1 or 2 days after infection. The enhanced response of spleen cells from *S. typhimurium* was observed before bacteria are normally detected in the livers and spleens of such animals. Therefore, the model of macrophage activation postulated above must be open to vast variations in time scale for its developement and the factors controlling this are not known, though the degree and timing of enhancement appears to be dependent on the number of bacteria given.

It was also found in the course of this work that macrophages from *M. lepraemurium* and *P. yoelii* animals at later stages of infection suppressed the *in vitro* antibody response of normal nonadherent cells to SRBC. This depression can be postulated to be mediated by several mechanisms, e.g. macrophage dysfunction, generation of suppressor T cells or antigen/antibody complexes on the macrophage surface preventing the binding of factor(s) necessary for the antibody response. The mechanism I prefer is that macrophages are malfunctioning, because at the height of infection they are full of bacilli or parasitised red cells. It would, therefore, seem impossible that they should be able to phagocytose

and present antigen in a normal manner. The other two mechanisms are not dismissed as of no importance, it just seems that the first is the easiest to envisage and to test. If one were to postulate a dysfunction of macrophages at the height of these infections, one could explain the suppression of the responses to DNP-POL and DNP-Ficoll as showing that these antigens also needed the presence of functional macrophages, though the number required being lower, and hence the response is only inhibited at late stages of the infection as in the case of M. lepraemurium, when all the macrophages are full of bacilli c.f. the work of Lee et al. (1976). This still does not fully explain the situation as seen in malaria infection. The possibility of other mechanisms, therefore, remains open to debate. The results from the carrageenan experiments would also appear relevant at this point. Pretreatment of animals with carrageenan lead to their spleen cells giving a depressed response to SRBC in culture. This depression was mediated by macrophages. The exact mechanism of this suppression is very difficult to explain. Carrageenan is reportedly toxic for macrophages though, superficially, experiments here did not bear this out. It is, however, possible that carrageenan is cytotoxic for these cells but that this stimulates the production of new cells from macrophage precursors in the bone marrow. These cells may be immunoincompetent, hence the depression of the in vitro response. Whether such a mechanism can be envisaged for the depressions observed in the response of spleen cells from infected animals is not known. It is, however, another possibility to be considered.

The number of mechanisms it is possible to postulate for any of these observations are many and various. Judicious use of the Marbrook system should however provide an answer to many of the possible mechanisms. It would be possible to use different cell populations and by recombining them in various ways to obtain further information as to the mechanisms involved in these effects observed when using spleen cells from infected animals in the in vitro responses to both T dependent and independent antigens.

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Defect of macrophage function in the antibody response to sheep erythrocytes in systemic *Mycobacterium lepraemurium* infection

THE need for macrophages for optimal antibody responses, both *in vivo* and *in vitro*, to certain antigens is now established[1,2] and this paper describes experiments which have used this requirement to demonstrate a deficiency of macrophage function in mice experimentally infected with the rodent leprosy bacillus, *Mycobacterium lepraemurium*. This organism is an obligate intracellular dweller and is found particularly inside cells of the macrophage series although in terminal infection other cell types may be invaded. Systemically infected mice characteristically show macrophages overloaded with bacilli, ever increasing numbers of granulomata and increasing spleno- and hepatomegaly[3]. There is an increase in the phagocytic activity of the spleen and liver at an early stage of infection (I.N.B. and V.S.S., in preparation) indicative of alterations in macrophage function and we report here experiments which show that the *in vivo* and *in vitro* antibody response to sheep erythrocytes (SRBC) is depressed at later stages of infection. The results indicate a defect of macrophage function.

Young adult CBA female mice were injected intravenously with 10^9 *M. lepraemurium* freshly isolated from the heavily infected livers of mice infected 4–6 months previously (I.N.B. and H. N. Krenzien, in preparation). At 11–15 weeks after infection, when the present experiments were carried out, the mice appeared clinically healthy but at autopsy showed gross enlargement of liver and spleen. Ziehl-Neelsen stain revealed the presence of numerous mycobacteria in macrophages of various tissues, particularly liver, spleen and bone marrow. Uninfected mice of the same age and sex were used as controls. The *in vivo* antibody response was measured in mice injected intravenously with either 5×10^6 or 10^9 SRBC. Four days later, the number of antibody producing cells (PFC) present in the spleen of each mouse was determined using the method of Cunningham[4]. The *in vitro* response of spleen cells cultured for 4 d according to the method of Marbrook[5] was measured in the same way.

The results given in Table 1 show that the antibody response *in vivo* was reduced 11 weeks after infection and even more so at 14 weeks. This depression was more pronounced in animals injected with the smaller number of SRBC. It seemed greater when the results were expressed as PFC per 10 spleen cells because there was a 4–5-fold increase in the cellularity of the spleens from infected compared with normal mice. Serum antibody levels were also reduced in infected mice (results not shown). Spleen cells from infected animals cultured *in vitro* in the presence of SRBC failed to generate the expected number of PFC, particularly at 14 weeks.

Further studies were carried out *in vitro* to obtain information about the functional status of macrophages and lymphoid cells in infected spleens. Cells from infected and control mice were fractionated into adherent (macrophage) and non-adherent (lymphocyte) populations and were then cultured in various combinations in the Marbrook system. The results are shown in Table 2. Cultures containing only non-adherent cells produced small numbers of PFC and no PFC were detected in cultures of peritoneal cells or adherent spleen cells (not shown in the table). When non-adherent cells from the spleens of normal mice were supplemented with normal peritoneal cells or adherent spleen cells (experiments 2 and 3), the PFC response was similar to that found in cultures of unfractionated normal spleen cells. This result confirms earlier findings[7,8] on the requirement for macrophages in the primary response to SRBC *in vitro*. On the other hand, when adherent cells from the spleens of mice infected 11 or 14–15 weeks before were used to supplement non-adherent normal mouse spleen cells (experiment 6) the number of PFC found in the cultures was similar to that found in cultures of unfractionated spleen from infected animals (experiment 1). This result suggested that macrophages from infected mouse spleens were defective in some respect or, alternatively, that they had an inhibitory effect on the function of lymphocytes. These possibilities were tested by adding normal mouse peritoneal cells to cultures of unfractionated spleen cells from normal or infected animals (experiment 4). Under these conditions, the PFC response of the cultures prepared from infected animals was substantially improved (compare experiments 1 and 4) indicating that the presence of infected macrophages did not greatly interfere with the PFC response provided that adequate numbers of normal macrophages were present.

Further evidence for the essentially normal function of the lymphoid cells of infected mice was obtained from experiments where non-adherent cells from infected mouse spleens were supplemented with either normal peritoneal cells or normal spleen adherent cells (experiments 2 and 3).

The results presented here indicate, in contrast to another report[9], that the antibody response to SRBC is depressed in mice systemically infected with *M. lepraemurium*. Furthermore, the defect was associated particularly with the macrophages of infected animals and not with their lymphocytes at the stages of infection studied. Our experiments do not indicate a direct contact effect by heavily infected macrophages or the release of a suppressive factor. The nature of the defect has yet to be established. Two

Table 1 Antibody response of mice, or spleen cells from mice, infected with *M. lepraemurium*

Parameter of response	Dose of SRBC	Control	Weeks after infection	
			11	14
In vivo				
$\log_{10}$ PFC per spleen	5×10^6	5.85±0.02	5.33±0.06	4.87±0.16
	10^9	5.97±0.04	5.76±0.07	5.28±0.03
PFC per spleen, geometric mean ($\times10^{-3}$)	5×10^6	703	214	75
	10^9	937	570	189
PFC per 10^6 spleen cells	5×10^6	6,051±391	605±100	204±83
	10^9	4,899±452	1,250±154	446±21
In vitro				
PFC per culture	—	2,086±142	1,246±57	290±39

Spleen cells were cultured in RPMI 1640 medium containing HEPES, bicarbonate and 5% foetal calf serum. The cultures contained 20×10^6 spleen cells and 2×10^6 SRBC. The values shown are means ± s.e. of four mice in each group or quadruplicate cultures of spleen cells pooled from two or three mice. The significance of difference between control and experimental groups, calculated using the two-tailed Student/Welch's *t*-test[6], was at the level of $P<0.005$ or higher.

Table 2 The *in vitro* antibody response of spleen cells from mice infected with *M. lepraemurium*

Experiment	Cells cultured	PFC per culture		
			Weeks after infection	
		Control	11	14–15
1	Spleen unfractionated	2,140±82	960±57	620±22
2	Spleen non-adherent+normal PC	2,140±85	1,790±78	1,466±33
3	Spleen non-adherent+normal spleen adherent	1,886±41	1,800±26	1,706±63
4	Spleen unfractionated+normal PC	2,600±10	1,716±51	1,510±59
5	Spleen non-adherent	190±12	96±46	80±34
6	Normal spleen non-adherent+infected spleen adherent	—	1,192±64	610±13

Cultures of unfractionated spleen cells contained 20×10^6 cells ml^{-1}. Non-adherent cells were obtained by incubating spleen cells (20×10^6 ml^{-1}) twice for 30 min at 37 °C on glass and the free cells in the suspension were then gently agitated and poured off and 1 ml put into each culture vessel. The adherent spleen cells were removed from the glass surface by means of a rubber policeman before which the surface was vigorously washed with fresh medium. Peritoneal cells (PC) were obtained by washing the peritoneal cavity of untreated mice. Cultures were supplemented with macrophages by adding 2×10^6–3×10^6 viable adherent spleen cells or 4×10^6 peritoneal cells. All cultures contained 2×10^6 SRBC. Values given are means±s.e. of quadruplicate cultures.

possibilities are being considered in our immediate experiments. First, that overloading of macrophages with mycobacteria may interfere with their ability to ingest, process and present other antigens in a normal manner[10], and second, a possibility associated with the massive antibody production against *M. lepraemurium* antigens during infection, that antigen–antibody complexes are formed and become bound to the surface of macrophages, thus blocking the sites for binding of the factor(s) produced by T lymphocytes which may be required for antibody production to SRBC. Whether or not macrophage defects of a similar nature occur in diseases of man where macrophage involvement is extensive, for example, leprosy, leishmania and malaria, requires further study. If they do, they could account, at least in part, for the immunosuppressed state often associated with such infections.

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The role of macrophages in the adjuvant effect on antibody production of *Corynebacterium parvum*

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SUMMARY

Spleen cells from mice pre-treated with *C. parvum* gave an enhanced *in vitro* antibody response to SRBC, but not to DNP–POL. This enhancing activity was associated with the adherent, but not the non-adherent spleen cell population and was found to be radioresistant. It is concluded that macrophages are directly involved in the adjuvant effect of *C. parvum* and the possible mechanisms of action are discussed.

INTRODUCTION

Administration of *C. parvum* to experimental animals has a marked adjuvant effect on antibody responses to various antigens, although cell-mediated immunological reactions have been found on several occasions to be depressed (reviewed by Scott, 1974). *C. parvum* is also a potent activator of the mononuclear phagocyte system, stimulating both cellular proliferation (Warr & Šljivić, 1974a) and phagocytic activity *in vivo* (Halpern *et al.*, 1964; Warr & Šljivić, 1974b). It has been proposed that the adjuvant effect of *C. parvum* on antibody production is mediated via these stimulated macrophages (Howard, Scott & Christie, 1973) and the experiments we describe here demonstrate this.

MATERIALS AND METHODS

Mice. CBA female mice aged between 3 and 9 months were used as donors of spleen cells. Experimental mice were injected intravenously with 0·2 ml of *C. parvum* suspension containing 1·75 mg dry weight of organisms per millilitre (Batch PX 289, Wellcome Research Laboratories, Beckenham, Kent, England) 5 days before their spleens were removed. Control mice of similar age were untreated.

Preparation and culture of spleen cells. The medium used throughout was RPMI 1640 with glutamine (Flow Laboratories Limited, Irvine, Scotland), containing 5% heat-inactivated foetal calf serum, 15 mM HEPES, 24 mM sodium bicarbonate, 125 mg/l ampicillin, 125 mg/l cloxacillin and 10,000 i.u./l gentamicin. Spleen cell suspensions were prepared by sieving through a fine metal sieve. The cells were washed once and resuspended at a concentration of 20×10^6/ml. The cells were fractionated into non-adherent and adherent by incubating 15–20 ml of the suspension in a 300-ml glass medical flask lying flat on its side. After 30 min at 37°C the flask was turned over and the cells incubated for another 30 min. The non-adherent cells were then gently agitated and poured off, after which the surface was washed vigorously with fresh medium and the adherent cells removed from glass by means of a rubber policeman. The non-adherent cell fraction obtained in this way contained approximately 70% of the original cell concentration.

The cells were cultured for 4 days in Marbrook chambers (Marbrook, 1967) in the presence of either 2×10^6 sheep erythrocytes (SRBC) or 0·1 μg DNP–POL (kindly provided by Dr M. Feldmann), in a volume of 1 ml. Unfractionated spleen cells were cultured at a concentration of 20×10^6/ml and non-adherent ones at

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a concentration of 13–14 × 10^6/ml. Adherent cells were added to some cultures at the rate of 2–3 × 10^6 viable cells.

For some experiments cells were irradiated *in vitro* with a dose of 600 R using a Siemens Stabilipan X-ray machine operating at 240 kV and 10 mA, with a target distance of 34 cm, 1 mm Cu filter and a dose rate of 91·1 R/min.

The antibody response in the cultures was measured in terms of plaque-forming cells (PFC) by the method of Cunningham (Cunningham & Szenberg, 1968), using either untreated SRBC or DNP-coated SRBC for the response to DNP–POL (Strausbach, Sulica & Givol, 1970). The results are given as means ± s.e. PFC/culture of quadruplicate cultures of spleen cells pooled from five to seven mice.

RESULTS

The results of three separate experiments using SRBC as antigen and one experiment using DNP–POL are summarized in Table 1, which has been subdivided into sections for ease of reference. The results of some control cultures are not included in the Table. These showed that the numbers of anti-SRBC PFC of unfractionated spleen cells cultured in the absence of SRBC were 104 ± 30 and 164 ± 47 for normal and *C. parvum*-treated mice, respectively. No PFC were found in cultures containing only adherent cells and either of the two antigens.

Experiments using normal spleen cells (section A) confirmed earlier findings that macrophages were required for the *in vitro* response to SRBC (Mosier, 1967; Sjöberg, Andersson & Möller, 1972), but not to DNP–POL (Diener, Shortman & Russell, 1970).

Cultures of unfractionated spleen cells from *C. parvum*-treated mice produced approximately three times the number of anti-SRBC PFC found in cultures of cells from untreated

TABLE 1. Effect of pre-treatment of mice with *C. parvum* on the antibody response of spleen cells *in vitro*

Section	Spleen cells in culture			Response to:			
				SRBC			DNP–POL
	Unfractionated	Non-adherent	Adherent	1	2	3	
A	Normal	—	—	2050 ± 71	2190 ± 110	2280 ± 50	1446 ± 64
	—	Normal	—	210 ± 13	140 ± 22	225 ± 22	1236 ± 81
	—	Normal	Normal	1861 ± 62	1676 ± 54	1776 ± 17	1200 ± 90
B	*C. parvum*	—	—	7200 ± 423	6780 ± 100		1450 ± 68
	—	*C. parvum*	—	290 ± 55	110 ± 24		1486 ± 62
	—	*C. parvum*	*C. parvum*		5856 ± 210		1566 ± 76
C	—	Normal	*C. parvum*	3310 ± 273	5536 ± 70		1616 ± 87
	—	*C. parvum*	Normal	1816 ± 59	1820 ± 17		1536 ± 43
D	Normal-X	—	—			125 ± 33	
	—	Normal-X	—			0	
	—	Normal-X	Normal-X			0	
	—	Normal-X	Normal			20 ± 8	0
	—	Normal	Normal-X			1700 ± 134	1230 ± 53
E	—	*C. parvum*-X	*C. parvum*		140 ± 20		0
	—	*C. parvum*	*C. parvum*-X		5520 ± 70		1656 ± 76
F	—	*C. parvum*	Normal-X		1726 ± 35		1566 ± 76
	—	Normal-X	*C. parvum*		40 ± 18		0
	—	*C. parvum*-X	*Normal*		106 ± 30		0
	—	Normal	*C. parvum*-X		5610 ± 138		1600 ± 110

Normal indicates spleen cells from untreated mice and *C. parvum* indicates spleen cells taken from pre-treated mice. The suffix X indicates that cells were exposed to 600 R X-irradiation *in vitro*. The results are given as means ± s.e. PFC per culture of quadruplicate cultures.

mice (section B *vs* A), thus demonstrating the adjuvant effect of *C. parvum*. Removal of adherent cells from *C. parvum* spleen cell suspensions reduced the PFC response to background level and reconstitution with these cells restored almost completely the enhanced response. The involvement of macrophages in the enhanced responce to SRBC was demonstrated in recombination experiments in which non-adherent and adherent spleen cell fractions from untreated and pre-treated mice were cultured (section C). The PFC response was enhanced in cultures containing normal lymphocytes and *C. parvum* macrophages, but not in those containing *C. parvum* lymphocytes and normal macrophages.

Experiments in which irradiated spleen cells or cell fractions were cultured in the presence of SRBC provided further evidence that the adjuvant property was associated with the adherent cell fraction from *C. parvum*-treated mice (sections E and F). A dose of 600 R, which abolished the response of unfractionated spleen cells and the response of the non-adherent cell fraction in the presence of unirradiated adherent cells, had no effect on the co-operative function of normal adherent cells or the enhancing activity of these cells after administration of *C. parvum*.

The PFC response to DNP–POL was not affected by the removal of adherent cells or by the pre-treatment of donor mice with *C. parvum*. This response was, however, completely abolished when the non-adherent spleen cells were irradiated.

DISCUSSION

The experiments described here are relevant to several aspects of the adjuvant action of *C. parvum* on antibody production:

(1) Spleen cells from animals pre-treated with *C. parvum* when cultured *in vitro* in the presence of SRBC, a thymus-dependent antigen (Claman & Chaperon, 1969), gave an enhanced antibody response. In this respect *C. parvum* is different from *Bordetella pertussis* and BCG vaccines which produced an enhancement of the response to SRBC only when these adjuvants were present in the cultures of spleen cells from adjuvant-primed mice (Maillard & Bloom, 1972).

(2) The adjuvant effect was associated with the adherent spleen cell population, presumably macrophages, and not with the non-adherent cells, i.e. lymphocytes. The finding that the adherent cells were radioresistant, in contrast to the non-adherent cells, supports the conclusion that they were macrophages (Sjöberg *et al.*, 1972). The experiments in which irradiated cells were used also suggest that administration of *C. parvum* did not confer the property of glass adherence upon the radiosensitive cells (i.e. lymphocytes) and that the ability of macrophages to enhance the antibody response was not associated with their proliferation, since this is radiosensitive (Warr & Šljivić, 1974a).

(3) The response to DNP–POL, a T cell-independent (Feldmann & Basten, 1971), and macrophage-independent (Diener *et al.*, 1970) antigen, was not influenced by the pre-treatment of spleen cell donors with *C. parvum* under the conditions of the present experiments. This would suggest that administration of *C. parvum* did not influence, directly or indirectly, the function of B lymphocytes, which is at variance with *in vivo* findings using other T cell-independent antigens (Howard, Christie & Scott, 1973).

Although these experiments demonstrate that macrophages play a crucial role in the manifestation of the adjuvant effect after administration of *C. parvum*, the mechanisms involved are not understood. On the basis of the known involvement of macrophages in other systems the following possibilities could be considered at present.

(a) Macrophages from *C. parvum*-treated animals may be exerting a potentiating effect on antibody production because they are directly stimulated by ingested *C. parvum* organisms and take up, degrade and present the immunogenic moiety of the antigen in a more efficient manner. A difference in antigen handling has been found, for example, in Biozzi

'high' and 'low' responder mice and this is thought to relate to the different ability of these animals to produce antibodies (Wiener & Bandieri, 1974).

(b) The effect of *C. parvum* on macrophages may be mediated by T lymphocytes as has been suggested in the case of other adjuvants (Allison & Davies, 1971; Maillard & Bloom, 1972), perhaps through a non-specific factor released by T cells (Waldmann, 1975), although it has been suggested that such a factor does not require macrophages for its function (Lefkovits *et al.*, 1975). The present results are not incompatible with this possibility, providing it is postulated that (i) the continuous presence of activated T cells is not required, since macrophages from *C. parvum*-treated animals enhanced the response of normal lymphocytes, and (ii) activated T cells cannot stimulate macrophages under culture conditions, since lymphocytes from pre-treated animals did not show enhancement in the presense of normal macrophages. However, *in vivo* studies have revealed that *C. parvum* had an adjuvant effect on the response to SRBC in T cell-deprived mice (Howard *et al.*, 1973) and the response to pneumococcal polysaccharide, a thymus-independent antigen (Howard *et al.*, 1973), but not on the response to SRBC in thymus-less (nude) mice (Warr & James, 1975). In addition, *C. parvum* stimulated the phagocytic activity in T cell-deprived mice (Woodruff, McBride & Dunbar, 1974) and generated macrophages cytotoxic to tumour cells both in these and nude mice (Ghaffar, Cullen & Woodruff, 1975).

(c) Macrophages stimulated by *C. parvum* may be exerting their effect on immunologically competent cells through some soluble factor(s). Although no evidence is available at present to support such a mode of action, it has been shown recently that macrophage products can stimulate or depress the proliferation of lymphoid cells *in vitro* (Calderon & Unanue, 1975). If a similar mechanism is to be postulated to explain the effect of *C. parvum* it will have to act on T lymphocytes, since the response to DNP–POL was unaffected.

Further experiments are now in progress in an attempt to elucidate which of the above possible mechanisms may be operative.

NOTE ADDED IN PROOF

Similar results, using peritoneal macrophages from *C. parvum*-treated mice, have recently been described (Wiener, E. (1975) *Cell. Immunol.* **19,** 1), and a difference in the handling of KLH by these cells has been found (Wiener, E. & Bandieri, A. (1975) *Immunology*, **29,** 265).

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