ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES

by

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Thesis presented for the Degree of Doctor of Philosophy
at the University of Edinburgh in the Faculty of Medicine.

1984

To my parents, Jack and Jean Dougherty, and my wife Shona, for all their help and encouragement.

DECLARATION

I confirm that all the work presented in this thesis was conceived, planned and executed by myself.

Signed.....

Date 9/9/84

ACKNOWLEDGEMENTS

I would like to thank Professor J.G. Collee and Professor D.M. Weir for allowing me the opportunity to conduct the work for this thesis in the Immunology Laboratory, Department of Bacteriology, University of Edinburgh Medical School.

I am extremely grateful for the help and advice of my supervisor Dr. Bill McBride. I enjoyed the three years we spent together and hope to overcome the geographical difficulties and keep in touch. I wish also to thank the other members of the Immunology Laboratory - John, Liz, Anne, Mike and Sarah for being such good company.

I am indebted to Carolyn Brown for typing this thesis in such a short time.

Finally, I would like to acknowledge the University of Edinburgh for providing financial support in the form of a Postgraduate Studentship.

SUMMARY

On the basis of Fc receptor expression and phagocytic activity, approximately 20-25% of the cells present within the highly immunogenic, methylcholanthrene-induced, murine fibrosarcoma FSA-R could be classified as macrophages. These cells did not express the Mac-1 antigen and were approximately 50% I-A^k-positive. The expression of I-A^k required the presence of mature T cells, and macrophages obtained from tumours grown in nu/nu hosts were I-A^k negative. Both the percentage of macrophages present within the tumour and I-A^k expression by these cells remained constant during the observed period of tumour growth and during serial passage of the tumour *in vivo*.

Macrophages were enriched from enzymatically disaggregated tumour cell suspensions by virtue of their capacity to adhere tightly to glass or plastic surfaces and the accessory cell activity of the adherent cell population was investigated in various well defined *in vitro* assay systems. Thus tumour-associated macrophages were shown to be fully capable of reconstituting the primary anti-CRBC PFC response of Sephadex G-10 passed normal spleen cells. This function required the presence of I-A^k-positive cells, and tumour-associated macrophages treated with anti-Ia serum plus complement or obtained from tumours grown in nu/nu hosts, were inactive. Tumour-associated macrophages were also able to supply the essential cell activity required for effective cooperation between antigen-

primed T_H cells and normal B cells in the generation of PFC responses in vitro. Finally, tumour-associated macrophages were found to secrete a soluble factor(s) which considerably enhanced the primary anti-CRBC PFC response of whole normal spleen cells. Cell separation studies indicated that this activity was primarily a function of large macrophages. Furthermore, since macrophages treated with anti-Ia serum plus complement, or obtained from tumours grown in nu/nu hosts were as efficient as normal tumour-associated macrophages at enhancing the response, it would seem that $I\text{-}A^k$-positive cells were not involved in this function.

The possible significance of tumour-associated macrophage accessory cell activity is discussed.

CONTENTS

CHAPTER I - INTRODUCTION

1.0 IN SITU TUMOUR IMMUNITY

Pathologists have long been aware that most human and animal tumours are infiltrated by significant numbers of normal host cells. Indeed the first reports documenting the presence of such cells appeared during the latter half of the 19th century (reviewed by Underwood, 1974). Subsequent careful histological examination of tumour sections by conventional light microscopy has produced a wealth of data confirming these early findings (reviewed by Underwood, 1974; Ioachim, 1976). However, it is only recently that extensive efforts have been made toward understanding the *in vitro* and potential *in vivo* significance of tumour-associated host cells. Interest in this area has developed following increasing evidence that systemic tumour immunity neither reliably reflects the clinical status of cancer patients nor necessarily correlates with their prognosis. Moreover, most immunotherapy trials based on an evaluation of systemic tumour immunity have been conspicuously unsuccessful (reviewed by Currie, 1980).

A large number of studies have demonstrated that cells isolated from the lymph nodes, spleen, peritoneal cavity or circulation of tumour-bearing animals can effectively destroy autologous tumour cells *in vivo* and *in vitro* (reviewed by Baldwin, 1981). A variety of different cell types have been implicated in such anti-tumour reactivity including activated macrophages, cytotoxic T lymphocytes (Tc), natural killer cells (NK) and granulocytes. However, there was no experimental

proof for the assumption which was made consciously or by implication in many of these studies, that the same effector mechanisms were operational at the site of primary tumour growth. Indeed, when comparisons were made between systemic and local or *in situ* tumour immunity, both quantitative and qualitative differences were usually found (Haskill *et al.*, 1978).

It is clear therefore that *in situ* tumour immunity constitutes a crucial variable in the equation describing the tumour-host relationship and it is not unlikely that any conclusion regarding this relationship based exclusively on an evaluation of systemic tumour immunity may be partially or completely incorrect. Moreover, without an understanding of the trafficking of cells into tumours and their potential anti-tumour influences, there is little hope that immunotherapy can ever play an effective role in the treatment of cancer.

Virtually every type of anti-tumour effector cell which has been detected systemically has also been found *in situ* in at least one tumour system (reviewed by Haskill, 1982). However, of the cells which are commonly found within tumours, the macrophage has perhaps stimulated most interest. Macrophages have been shown to play a pivotal role in the induction, regulation and expression of both humoral and cell mediated immune responses and their presence within the tumour mass suggests various ways in which they may interact with the neoplasm.

2.0 MACROPHAGE HETEROGENEITY

Macrophages perform a large number of diverse and on occasion mutually antagonistic functions. They do for instance on one hand constitute a major line of host defence against the establishment and spread of microbial (Mackaness, 1964; Ruskin et al., 1969; Steigbigel et al., 1974) and neoplastic (Adams and Snyderman, 1979; Adams, 1982; Adams et al., 1982; Zoler, 1983) disease, while on the other hand they may support the growth of certain microorganisms (Mackaness, 1971) and tumour cells (Noar, 1979; Gablzon et al., 1980; Gorelik et al., 1982; Prehn, 1982). Similarly, macrophages act as accessory cells in the induction of humoral and cell mediated immune responses (Unanue, 1972, 1981; Howie and McBride, 1982a)but may also inhibit the proliferation and differentiation of lymphocytes (Kirchner et al., 1975a; Baird and Kaplan, 1977a,b) and other cells (Jones et al., 1975; Keller, 1976). Other functions ascribed to macrophages include the secretion of various biologically active substances (Nathan et al., 1980; Adams, 1982) and numerous homeostatic functions unrelated to host defence such as the removal of senescent red blood cells, turnover of lung surfactant and bone remodelling and resorption (Douglas, 1980; Silverstein, 1982). The range of capabilities is in fact so diverse as to raise the question of whether one cell can possibly perform all these functions or whether as is the case with the T cell system, distinct subsets of macrophages exist each capable of performing only certain functions.

2.1 Identification and characterization of macrophage heterogeneity

Although all macrophages show common morphological,biochemical or functional characteristics they are by no means uniform in these respects. As long ago as 1925 Sabin et al. using supravital staining techniques described the similarity between resident murine macrophages in the spleen, peritoneal cavity and liver and pointed out that such cells differ in many respects from monocyte-derived exudate macrophages elicited by intraperitoneal (i.p.) administration of blood. The development in recent years of various assays of macrophage function, the identification of cell surface and intracellular antigens using heteroantisera and monoclonal antibodies, and the use of assays which identify and quantitate intracellular and extracellular enzyme activities, has produced a wealth of data documenting macrophage heterogeneity (reviewed by Walker, 1976; Hopper et al., 1979; Walker, 1982). Thus it seems that for almost every characteristic which has been examined macrophages show diversity of expression.

Two major types of macrophage heterogeneity have been described. The first is termed "Interpopulation" heterogeneity and refers to differences between macrophage populations obtained from different tissue sites. The second is termed "Intrapopulatior heterogenetiy and refers to differences between subpopulations of macrophages obtained from within a particular site (Walker, 1982).

It is relatively easy to speculate on the possible mechanisms

by which such macrophage heterogeneity could be generated but somewhat more difficult to find good experimental evidence to support proposed mechanisms. What little evidence there is available suggests that macrophage heterogeneity results largely from two main mechanisms - differentiation and modulation - although the issue is further complicated by the proposed existence of macrophage sublineages derived from distinct bone marrow precursors.

2.2 Differentiation

It is now generally accepted on the basis of a large body of experimental evidence, that under normal steady state conditions, tissue macrophages originate mainly from multipotential haematopoietic stem cells located in the bone marrow (reviewed by van Furth, 1981). These cells divide and differentiate giving rise to a population of cells which are heterogeneous in nature and variably committed to produce cells of both the granulocyte and macrophage series (GM-CFC) (Metcalf and McDonald, 1975; Byrne *et al.*, 1977; Bol and Williams, 1980). The most immature cell so far recognized which is entirely committed to macrophage production is the monoblast (Goud and van Furth, 1975; Goud *et al.*, 1975). Murine monoblasts have a cell cycle time of about 12 hours (van Furth, 1981) and division of one cell produces two promonocytes which in turn divide after approximately 16 hours to give two monocytes (van Furth, 1981). These proliferation and differentiation

events are absolutely dependent, at least *in vitro*, upon the presence of a colony stimulating factor (CSF) known as CSF-1 (Metcalf *et al.*, 1980; Watson and Prestidge, 1983). CSF-1 appears to be a largely proliferative signal and distinct differentiation signals (D factors) are probably also involved in the sequence of events (Onozaki, *et al.*, 1983). Monocytes do not remain in the bone marrow for long and are rapidly liberated into the circulation (van Furth, 1981). Their half-life in the blood is about 17 hours (van Furth, 1981) and they are thought to migrate into the tissues where they further differentiate to form tissue macrophages (Blusse and van Furth, 1979).

However, the generation of macrophages may be more complex than this. A number of studies have demonstrated that a small proportion of macrophages isolated from sites, in addition to the bone marrow, such as the peritoneal cavity, blood, lung, liver, thymus and lymph nodes are capable of generating colonies *in vitro* in the presence of colony stimulating factors (Lin, 1977; MacVittie and McCarthy, 1977; McCarthy and MacVittie, 1978; MacVittie and Provaznik, 1978; Chen *et al.*, 1979). Unlike GM-CFC such cells can survive the initial absence of CSF, only proliferate *in vitro* after a delay of one to two weeks, and generate small colonies composed solely of macrophages (Metcalf, 1982). These macrophage colony forming cells (M-CFC) are more mature than bone marrow GM-CFC

in that they are already committed entirely to macrophage production.

The quantitative significance of local proliferation in the generation of tissue macrophages *in vivo* is the subject of considerable debate (van Furth, 1981; De Bakker and Daems, 1981). The percentage of macrophages which incorporate ^{3}H-thymidine *in vivo* varies from one tissue to another. For example, only approximately 5% of peritoneal macrophages incorporate the label (van Furth, 1978), while in the lung, this figure may be as high as 20% (Lin *et al.*, 1975; Chen and Lin, 1982). Consequently, it seems likely that the extent to which local proliferation contributes to the macrophage pool will depend largely on which tissue is being examined. The nature and origin of these proliferating cells remains to be established although they are probably local M-CFC.

The macrophages which accumulate at inflammatory sites are also derived mainly from blood monocytes (Bursuker *et al.*, 1982). However, depending on the nature of the inflammatory stimulus used, a variable proportion of such cells may proliferate *in situ* (Dienstman and Defendi, 1978). Nevertheless, it seems unlikely given the magnitude of the monocyte influx that local proliferation could contribute much numerically to the total macrophage content of inflammatory sites at least early in the response.

While certain macrophage characteristics are stably expressed during differentiation from bone marrow precursors, the expression of others depends upon differentiation stage and may vary in both a qualitative and quantitative manner. For example, during the differentiation of macrophages from bone marrow precursors *in vitro*, the expression of receptors for C3b, the ability to pinocytose horse radish peroxidase and lysozyme activity all increase (Goud and van Furth, 1975; Goud *et al.*, 1975). Similarly, Bursuker *et al.* (1982) have suggested that the expression of the lysosomal enzyme β-galactosidase may be used to determine macrophage differentiation stage. It seems that the enzyme activity increases during differentiation but unlike many other characteristics (e.g. C3b expression) β-galactosidase levels are unaltered by various environmental stimuli (Bursuker *et al.*, 1982).

As they differentiate macrophages may also express different functional characteristics. For example, the cells exhibiting natural killer-like activity isolated from a *Corynebacterium parvum* - elicited peritoneal exudate or generated *in vitro* during bone marrow culture were identified as promonocytes while more mature cells did not perform this function (Lohmann-Matthes *et al.*, 1981).

Differentiation of monocytes into characteristic tissue macrophages *in vivo* or *in vitro* is accompanied by a considerable

increase in cell size (Lee, 1980). It is possible to exploit this fact to separate macrophages into functionally distinct subpopulations and thereby demonstrate a correlation between differentiation stage and functional capability (Lee et al., 1979; Lee, 1980). For example, only small, presumably poorly differentiated, macrophages expressed Ia determinants and presented macrophage-bound antigen to primed T cells in an antigen-specific proliferation assay. It was proposed that as macrophages differentiate they lose Ia expression and accessory cell function (Lee et al., 1979; Lee, 1980).

These findings suggest that as macrophages differentiate they may express a series of functionally distinct phenotypes. It is possible therefore that differences in macrophage differentiation stage may contribute to interpopulation and intrapopulation heterogeneity. Within a particular macrophage population it is likely that some cells are poorly differentiated having just arrived from the blood stream while others may have been resident for some period of time. Inflammatory agents produce dramatic alterations in the functional characteristics of a macrophage population and it is possible that they do this in part by causing an influx of relatively immature cells into a site thereby altering the proportion of cells in different differentiation stages (Bursuker et al., 1982). Proliferation of immature exudate macrophages may further add

to the degree of heterogeneity. It has been reported that the expression of several cell surface components varies with the cell cycle (Bursuker and Goldman, 1982, 1983). The heterogeneity of Fc receptor expression in the macrophage tumour cell line $P388D_1$ and other cell lines may be due to cell cycle variation (Gandour and Walker, 1983). Neuman and Sorg (1981) have suggested that macrophages express distinct functions at different stages in the cell cycle and that proliferation may contribute to macrophage heterogeneity.

2.3 Modulation

The term "modulation" is used to describe those induced changes in macrophage morphology, biochemistry or function that result from continuous exposure to a particular signal; when the signal is removed the changes are reversed and the cell rapidly returns to a quiescent state, (Dougherty and McBride, 1984).

Macrophages from different tissues differ considerably with respect to a large number of characteristics and modulation is assumed to be the major source of such interpopulation heterogeneity. The implication is that the cells entering different tissue sites are essentially identical and that all the events which lead to the generation of interpopulation heterogeneity occur entirely within a site as a result of exposure to modulating signals. For example, alveolar macro-

phages function in a high oxygen environment, are largely aerobic and generate their energy by oxidative phosphorylation, while peritoneal macrophages function in a low oxygen environment, are largely anaerobic and generate their energy by glycolysis. Consequently, the two populations differ in their expression of key enzymes of the oxidative phosphorylation and glycolysis metabolic pathways (Oren et al., 1963; Karnovsky et al., 1970; Simons et al., 1977). Bar-Eli et al. (1981) have shown that the progeny of single bone-marrow macrophage precursors exhibit different metabolic enzyme profiles depending upon culture conditions. If cultured in a high oxygen environment they expressed the enzymes of oxidative phosphorylation and if cultured in a low oxygen environment they expressed the enzymes of glycolysis. These environmentally induced changes were reversibly acquired and when cells adapted to low oxygen tension were exposed to high oxygen conditions there was a time dependent recovery in the expression of the enzymes of oxidative phosphorylation.

In as much as enzyme expression is readily altered by the cellular environment it is a modulated characteristic. Many other aspects of interpopulation heterogeneity may be the result of environmental modulation. Macrophage Ia expression (Section 3.3) and activation status (Section 4.1) are two obvious examples. Although a feature of modulated changes is

that they are reversible, it is possible to imagine a situation where modulating signals exert such a profound effect on cellular physiology that some induced characteristics are more permanently acquired.

In addition to the effect of continuous exposure to normal microenvironmental stimuli, the expression of a number of important macrophage characteristics may also be altered by non-microenvironmental stimuli. For example, the degree of macrophage heterogeneity which results from the administration of inflammatory stimuli could be explained in terms of the nature of the modulating signals generated and the responsiveness of macrophage subpopulations to these signals. Macrophage responsiveness is likely to depend upon differentiation stage and previous exposure to modulating signals thereby generating heterogeneity.

2.4 Sublineages

The possible existence of true sublineages of macrophages derived from distinct bone marrow precursors provides an additional level of complexity to the subject of macrophage heterogeneity. These precursors may produce cells with a preferred tissue destination and functional capabilities, and stimuli such as those generated by inflammation may lead to the preferential involvement of certain sublineages.

The evidence in support of the existence of distinct macrophage sublineages is not conclusive. However, it appears that macrophage precursor cells are inherently heterogeneous with respect to physical, biochemical and functional characteristics (Metcalf and McDonald, 1975; Byrne et al., 1977; Bol and Williams, 1980; Nicola et al., 1980). Moreover, colonies of macrophages clonogenically derived from individual bone marrow precursors may express different phenotypes. For example, Claesson and Olsson (1980) found that only selected colonies of mouse bone marrow-derived macrophages, expressed strong natural cytotoxicity for tumour targets. The authors did not however examine the differentiation stage of the cells in each colony. Previously, promonocytes have been identified as the cells responsible for natural cytotoxicity (Lohmann-Matthes, 1981) and in this connection it is interesting to note that colonies consisting exclusively of promonocytes have recently been described in cultures of mouse bone marrow (Buhles, 1979).

Bursuker and Goldman (1982) have shown that in cultures of bone marrow from normal mice, macrophage clones exclusively express either high or low 5'-nucleotidase activity. The expression of the ectoenzyme 5'-nucleotidase has proven a useful marker to distinguish resident and inflammatory peritoneal macrophages. The resident population express high 5'-nucleotidase activity whereas inflammatory macrophages express low

activity (Bursuker and Goldman, 1982, 1983). These results have been taken to suggest that resident and inflammatory macrophages may arise from distinct bone marrow precursors (Bursuker and Goldman, 1982). This conclusion was reinforced by the finding that in cultures of bone marrow from animals undergoing an inflammatory reaction, the percentage of macrophage clones expressing low 5'-nucleotidase activity was significantly increased (Bursuker and Goldman, 1983). In this instance, there appeared to be no major differences in the degree of differentiation between bone marrow-derived macrophages from normal or inflammation bearing animals as determined by the expression of β-galactosidase, although clonal expression of this enzyme was not similtaneously examined.

Thus the progeny of individual macrophage precursors appear to retain certain distinctive characteristics during differentiation in vitro. Assuming that there is no internal regulation within a clone which results in collective expression of particular characteristics, then these experiments show the existence of distinct macrophage sublineages. The actual number of such sublineages remains to be determined, although it seems unnecessary to postulate that many will be found and heterogeneity is likely to be generated mainly by differentiation and/or modulation of one, or at the most, a few highly versatile macrophage lineages.

3.0 MACROPHAGES AND THE AFFERENT ARM OF THE IMMUNE RESPONSE

The immune system is a complex collection of cells and soluble mediators capable of recognizing and rapidly responding to challenge with a large number of different foreign molecular configurations (antigens) in one or more of a series of specific ways. However, an immune response does not result from a simple encounter between immunocompetent lymphocytes and antigen. On the contrary, both the nature and the magnitude of the response generated following challenge with antigen depends upon a complex cascade of well controlled interactions involving many different cell types. The initial step in the reaction cascade leading to the generation of an effective immune response to most, if not all, antigens requires cooperation between immunocompetent helper T lymphocytes (T_H cells) and various non-lymphoid accessory cells (reviewed by Unanue 1972, 1981). Although our understanding of this interaction is far from complete, it is evident that the activation of populations of T_H cells requires that accessory cells "process" the antigenic material and "present" the appropriate epitopes to T cells in association with major histocompatibility complex (MHC) encoded class II glycoproteins (Ia antigens) (reviewed by Unanue, 1981; Unanue *et al.*, 1984).

Many diverse cell types have been shown to possess this accessory cell activity including macrophages (reviewed by

Unanue, 1981), Langerhans cells (Silberberg-Sinakin et al., 1976; Stingl et al., 1978; Braathen and Thorsbay, 1980), dendritic cells (Nussenzweig et al., 1980; Steinman et al., 1981; Britz et al., 1982; Miyazaki and Osawa, 1983), follicular dendritic cells (Klaus et al., 1980), veiled cells (Knight et al., 1982), interdigitating cells (Humphrey, 1981), B lymphocytes (L'Age-Stehr, 1981; Chesnut et al., 1982), endothelial cells (Hirschberg et al., 1980), and neutrophils (Okuda et al., 1980). The functional cells always expressed Ia determinants and their activity was generally held to be MHC restricted. What is not clear at present, is whether all these different accessory cells present antigen in the same way and stimulate the same type of response. In this connection it is interesting to note that Ramila et al. (1983) have recently reported that while Ia-positive tumour cell lines and peritoneal exudate cells were both able to induce MHC-restricted antigen-specific T cell proliferation, only the peritoneal cells were able to induce functional antigen-specific T_H cells. Furthermore, the type of Ia molecules expressed by the accessory cell may be important. Thus while presentation of antigen by an I-A positive accessory cell seems to be critical for T_H cell activation (reviewed by Unanue, 1981), recent data has suggested that I-J positive accessory cells are required for the activation of antigen-specific T suppressor cells (T_S cells) (Gershon, 1974; Minami et al., 1982; Zembala

et al., 1982; Lowy et al., 1983, 1984). It is likely therefore that in mixed cell cultures antigen presentation may be carried out by more than one cell type (Miyazaki and Osawa, 1983) and the overall response generated may depend to some extent on the number of different antigen presenting cells that are present.

Although antigen presentation represents the initial step in the generation of an effective immune response, the subsequent clonal expansion and differentiation of immunocompetent lymphocytes requires the involvement of various lymphostimulatory factors (reviewed by Howie and McBride, 1982a)and expression of this function probably varies between different accessory cell populations (Miyazaki and Osawa, 1983). In addition, although Ia-negative cells cannot present antigen, they may nevertheless secrete lymphostimulatory factors (Lipsky and Kettman, 1982) and may thus significantly contribute to the generation of an immune response.

3.1 Antigen processing by macrophages

When antigen enters a lymphoid tissue it is taken up by macrophages lining the sinuses or dispersed among the lymphoid elements. Many studies have demonstrated that antigen associated with macrophages plays a key role in the initiation of the immune response by lymphoid cells (reviewed by Unanue

1972, 1981). Most of the antigen internalized by macrophages is rapidly catabolised, but a small percentage (10-20%) is broken down more slowly (Unanue and Askonas, 1968) being retained either as a component of the macrophage surface (Unanue and Cerrottini,1970) or in an internal pool (Ellner and Rosenthal, 1975). Some antigenic components may be released from the macrophage as soluble products (Schmidtke and Unanue, 1971; Cruchaud et al., 1975).

For most antigens, a processing step is required before T_H cells can recognised and respond to them. For example, the proliferation of primed T lymphocytes in response to Listeria monocytogenes requires that macrophages bind and internalize the bacteria, and process the antigen in an acid vesicle (Ziegler and Unanue, 1981, 1982). Interference with any of these steps blocks presentation. A similar obligitory antigen processing step has been described for soluble Listeria polypeptides as well as other well-defined proteins (Chesnut et al., 1982; Lee et al., 1982). It should be noted however that transfer of antigen from one cell to another may occur (Ellner et al., 1977) suggesting that processing and presentation need not necessarily be carried out by the same cell. In this connection,Shimonokevitz et al. (1983) have reported that a small tryptic peptide from ovalbumin can be presented by fixed macrophages. Thus once a protein is fragmented it is apparently

in a form which is able to interact with membrane structures on a presenting cell in such a way as to be recognised by the T cell.

The question of which epitopes are displayed by antigen presenting cells is an important one with respect to T and B cell repertoires. It has been suggested that T cell reactivity can be directed against the primary sequence of partially digested or unfolded proteins while B cells can recognize only native confirmational antigenic determinants (Sela, 1969). Although this phenomenon requires further examination it is possible that such differences in functional T and B cell repertoires could in part be a reflection of the form in which antigen is presented to the different lymphocyte populations.

3.2. Antigen presentation by macrophages

Many early studies indicated the highly immunogenic nature of macrophage-associated antigen *in vivo* and suggested a requirement for adherent phagocytic cells in order to generate immune responses *in vitro* (reviewed by Unanue, 1972). However, the essential nature of macrophage antigen presentation was not fully appreciated until the discovery of the restriction imposed on cellular interactions by products of the MHC.

The first observations on the histocompatibility requirements of macrophage-T lymphocyte interactions were published by Alan Rosenthal and Ethan Shevach in 1973 (Rosenthal and Shevach, 1973; Shevach and Rosenthal, 1973). Making use of two inbred strains of guinea pig which differed at MHC loci, strains 2 and 13, they investigated the ability of antigen-pulsed macrophages from one strain to stimulate the proliferation of antigen-primed T lymphocytes from the other strain. It was found that antigen-induced T cell proliferation was only obtained when the macrophages and T cells came from the same strain. If the macrophages and lymphocytes came from different strains then no proliferation was seen above the control values obtained using macrophages that were not pulsed with antigen. The possibility that the lack of responsiveness found with allogeneic combinations of T cells and macrophages was the result of a suppressor effect was ruled out by the finding that T cells from an F_1 cross ($(2x13)F_1$) could respond

perfectly well to antigen in the context of either strain 2 or strain 13 macrophages. It was concluded from these results that identity at MHC loci was required for effective macrophage-T lymphocyte interaction in the generation of a proliferative response. Subsequent studies indicated that the major differences between the MHC of strain 2 and strain 13 guinea pigs were found within the I-region (Geczy et al., 1975; Schwartz et al., 1976), suggesting that the MHC genes restricting macrophage T cell interaction also lie within this region. This conclusion is strongly supported by the finding that macrophages and T cells from outbred guinea pigs could only interact if the two populations were histocompatible at the I-region (Shevach, 1976).

These initial findings in the guinea pig have been confirmed and extended in a wide variety of species including man. In murine systems, the availability of genetically well defined congenic mouse strains with intra-H-2 recombinations has enabled the loci controlling macrophage-T cell interactions to be mapped more precisely. For most antigens the histocompatability requirement mapped to the I-A subregion (Yano et al., 1977, 1978; Schwartz et al., 1978), although macrophages and T cells having only I-E/C identity were capable of generating proliferative responses to some antigens (Richman et al., 1980), while for other antigens, the responding T cells and macrophages were required to share both I-A and I-E/C

subregion homology (Rosenwasser and Rosenthal, 1979; Schwartz et al., 1979).

The requirement for I-region identity between T cells and antigen pulsed macrophages does not only apply to proliferative responses. Transfer of delayed type hypersensitivity (DTH) by antigen-primed T cells required that recipient and donor share I-A subregion homology, presumably reflecting a requirement for antigen presentation at the site of antigenic challenge in order to restimulate the primed T cells (Miller et al., 1977; Vadas et al., 1977; Smith et al., 1979). Erb and Feldmann (1975a) found that the induction of T_H cells for antibody formation required that the antigen pulsed macrophages and T cells were histocompatible at I-A. Interestingly, T_H and B cells also had to share I-A subregion identity for successful co-operation (Katz and Benacerraf, 1975; Sprent, 1978a). The interaction of T cells with antigen on macrophages and on specific B cells may thus be identical or very similar. Finally, in some experimental systems T_S cell responses have been shown to be MHC restricted. When observed such restrictions map either to the I-J subregion (Tada et al., 1977) or to H-2D (Miller et al., 1978).

Several theories have been put forward in an attempt to explain MHC restriction. Early models envisaged a like-like form of interaction in which identical MHC-encoded

molecules on the surface of a macrophage and a T cell became physically associated (reviewed by Schwartz, 1984). Responding T cells were proposed to have an antigen receptor which imparted specificity. Presumably both molecular interactions had to occur at the same time in order to stimulate the T cell to respond. However, several lines of evidence argue against the like-like interaction model and suggest instead that MHC restriction is an inherent property of T cell recognition of specific MHC determinants on the surface of an antigen presenting cell. For example, although T cell proliferation was effectively inhibited by pretreatment of antigen-pulsed macrophages with antisera or monoclonal antibodies directed against Ia determinants, preincubation of T cells with anti-Ia antibodies had no effect (Schwartz et al., 1978; Thomas et al., 1978; Lerner et al., 1980). This finding is consistent with the observation that in some species, notably the mouse, the responding T cells did not bare detectable Ia molecules (reviewed by Unanue, 1981). Using a different approach, studies on F_1 hybrids between two histoincompatible stains have shown that these animals contain two populations of primed T cells both of which respond to the same antigen although each does so only in the context of one set of parental class II antigens and not the other (Miller et al., 1976; Paul et al., 1977; Sprent, 1978b). Since I-region genes are co-dominantly expressed it is clear that if a like-like interaction occurred then it would have been expected

that F_1 T cells primed against an antigen in the context of one parental class II antigen would be able to respond to the same antigen if presented in the context of the other parental class II antigen. The possibility that a suppressor mechanism may account for these results has recently been ruled out by the isolation of T cell clones from F_1 animals which respond to antigen only if it is presented by macrophages from one parental strain and not the other (Sredni and Schwartz, 1981). Since these clones have been purified away from other T cells, particular T_S cells, it appears no longer possible to attribute MHC restriction of the macrophage-T cell interaction to any type of suppressor cell effect.

The conclusion that may be drawn from these results is that an explanation of MHC restriction by a like-like interaction model is untenable. Instead the T cell must possess a different molecule, presumably a recognition structure which is complementary to the Ia molecule expressed on the macrophage surface.

The potent inhibitory effect of anti-Ia antibodies on T cell responses contrasts sharply with the lack of inhibition usually produced by antibodies against native antigen (Ellner and Rosenthal, 1975; Ben-Sasson et al., 1977; Thomas and Shevach, 1978; Lerner et al., 1980). However, recently Lamb et al. (1984) reinvestigated the effect of anti-antigen

antibodies on presentation using human T_H cell clones recognizing a 24 amino acid synthetic peptide (termed p20) derived from the influenza haemagglutinin-1 molecule. In this highly refined system, it was found that antibodies raised against p20 could inhibit the proliferative response of the T cell clones. Inhibition was possible either by treating antigen-pulsed macrophages with antibody prior to using them to stimulate the cloned T cells or by coculturing p20 antigen and antibody to p20 with cloned T cells and macrophages. Berkower et al. (1983) using a similar experimental system found that the response of T cell clones to sperm whale myoglobin was inhibited by monoclonal antibody directed against the epitope recognised by the T cell clone.

The reason why these studies succeeded in demonstrating inhibition of T cell responses by antibody against native antigen while so many previous attempts had failed is unknown, although the answer probably lies in the use of cloned T cells, small well-defined antigens and high titre monoclonal antibodies known to react with an antigenic determinant identical or very near that recognized by the T cells. Whatever the case may be, it would nevertheless seem that in addition to Ia, T cells also recognize antigenic determinants on the surface of a presenting cell. What then is the nature of the association between Ia and antigen?

Several studies have suggested that a direct physical association between antigen and Ia molecules is required for antigen presentation. Rock and Benacerraf (1983a,b) found that the presentation of the antigenic copolymer L-glutamic acid-L-alanine-L-tyrosine (GAT) could be competitively inhibited by the structurally related copolyer L-glutamic acid-L-tyrosine (GT). This affect was strongly influenced by the MHC allele of the presenting cell. Thus presentation of GAT by a cell expressing I-A^d was effectively blocked by GT while presentation by a cell expressing I-A^b was not. The conclusion drawn from these studies was that antigen physically associates with Ia determinants on the surface of a presenting cell. The GAT association site on I-A^d cells may be blocked by GT while the similar site on I-A^b can not.

Recently, further studies were undertaken in order to more directly test this model of antigen presentation (Rock and Benacerraf, 1984). It was argued that the ability of GT to inhibit presentation of GAT by I-A^d cells but not by I-A^b cells suggests that the GAT association site on these two cells may differ and as a result it should be possible to generate alloreactive H-2^b clones which recognize the pututive GT/GAT interaction site on I-A^d. T cell hybridomas were derived from B10 (H-2^b) T cells stimulated, in a primary mixed lymphocyte culture (MLC) with BALB/c (H-2^d) stimulators and screened for IL-2 production in response to BALB/c cells.

Alloreactive T cell hybrids were identified, which as expected, demonstrated a clonal specificity for I-A^d or I-E^d. The response of these hybrids to I-A^d-bearing stimulator cells was tested in the presence or absence of antigen. It was predicted that if antigen associated with Ia, the response of the T cell hybrids would be inhibited either as a result of determinant blocking or some other focal change in Ia structure (e.g. conformational change). This was indeed the result which was obtained. A small percentage of the alloreactive T cell clones were inhibited in a dose dependent fashion if the stimulator cells were pulsed with GAT. Moreover, there was a close correlation between the selective loss of allostimulatory activity and the ability of these cells to present GAT. Similar results were obtained using GT which specifically competes with GAT for presentation in association with I-A^d.

The most likely interpretation of these findings is that macrophages present a complex of antigenic determinants and Ia to T cells for the generation of an effective immune response. Several other observations substantiate this general conclusion. For example, Erb and Feldmann (1975b) have described a factor containing both Ia and antigen that was released from antigen-pulsed macrophages and was able to trigger antigen-specific T cells. More recently, Puri and Lonai (1980) implicated a similar factor in the activation of T_H cells.

3.3 Regulation of macrophage Ia expression

Since T cells recognize antigen only in conjunction with Ia it follows that the regulation of Ia expression on the antigen presenting cell may well be a critical step in the control of immune responsiveness. Indeed, Beller (1984) demonstrated a stoichiometric relationship between macrophage Ia expression and accessory cell activity. A similar correlation was found using splenic accessory cells with genetically determined quantitative differences in Ia expression (Matis et al., 1982).

The percentage of macrophages expressing Ia determinants varies from tissue to tissue, although in the absence of external stimuli, the figure for a given tissue is remarkably constant, (reviewed by Unanue, 1981). The regulatory mechanisms that determine this basal level of macrophage Ia expression are not clear. Signals from mature T cells or microbial stimulation are probably not involved, since the same percentage of Ia positive cells are found in the tissues of athymic (Lu et al., 1981) and pathogen free mice (Unanue et al., 1984). However, it is evident that Ia is associated mainly with young recently emigrated macrophages and unlike the expression of MHC class I molecules, the expression of Ia is a transient event (Beller and Unanue, 1981). Studies on Ia expression by peritoneal macrophages revealed a progressive loss with

time in culture so that by 72 hours most of the cells no longer expressed Ia (Beller and Unanue, 1980). Such a decrease could not be accounted for by selective loss of macrophages from the culture dish. Similarly, in vivo whole body X-irradiation resulted in a rapid decrease in the percentage of peritoneal macrophages which expressed Ia (Beller and Unanue, 1981). The kinetics of this loss was very similar to that found in culture and could not be attributed to the selective disappearance of a subset of macrophages from the peritoneal cavity. Presumably, irradiation prevents the generation of macrophage precursors in the bone marrow and as macrophages in the tissue gradually loose Ia expression the number of Ia positive cells is no longer maintained by an influx of young cells.

Macrophage Ia expression is also under both positive and negative regulatory control. For instance the phagocytosis of latex beads, killed Listeria monocytogenes organisms, opsonized erythrocytes or soluble antigen-antibody complexes caused Ia-positive macrophages to synthesize new Ia determinants and prevented the subsequent loss of the antigen during culture (Beller and Unanue, 1980). Interestingly, only cells which were already Ia-positive could be stimulated to synthesize Ia by particle uptake. If macrophages expressing Ia were eliminated with antibody and complement the remaining Ia-negative cells did not synthesize Ia even after particle phagocytosis. In contrast, immature Ia-negative macrophages could be induced

to express Ia by exposure to lymphokines *in vitro* (Steeg *et al.*, 1980; Steinman *et al.*, 1980; Beller and Ho, 1982) or *in vivo* (Scher *et al.*, 1980, 1982). The production by T cells of lymphokines that induce macrophage Ia expression takes place after activation by antigen-bearing Ia-positive macrophages (Scher *et al.*, 1980) and is a clear example of the functional interdependence of these two populations of cells. There is convincing evidence that γ-interferon has Ia-inducing activity (Steeg *et al.*,1982; King and Jones, 1983; Beller, 1984) but whether this is the only lymphokine with this activity remains to be determined.

Macrophage Ia expression can also be actively down regulated by a number of inhibitory factors including prostaglandins (Snyder *et al.*, 1982) and α-fetoprotein (Lu *et al.*, 1984). Thus the expression of Ia by macrophages in normal tissues may be controlled by local inhibitory factors. It is interesting to note that i.p. injection of indomethacin produced a considerable increase in the percentage of Ia-positive peritoneal macrophages (Snyder *et al.*, 1982) suggesting the involvement of prostaglandins in the control of Ia expression by these cells *in vivo*.

3.4 Differentiation and clonal expansion of T_H cells

The interaction of T_H cells with antigen presented in conjunction with Ia determinants represents the initiating event

in T cell activation. However, the subsequent development of functional activity requires the involvement of various lymphostimulatory factors, produced by appropriately stimulated macrophages and lymphocytes, which induce clonal expansion and differentiation of the responding cells (reviewed by Howie and McBride, 1982a).

The best studied macrophage-derived lymphostimulatory factor is interleukin 1 (IL-1) (reviewed by Oppenheim and Gery, 1982). Resting macrophages secrete only small and variable amounts of IL-1 but this basal level of secretion may be rapidly increased following interaction with a wide variety of different stimuli (Calderon et al., 1975; Unanue et al., 1976). A particularly relevant stimulus is provided by immune T cells upon interaction with macrophage-bound antigen. The secretion of IL-1 required not only the presence of Ia-bearing macrophages but also that the macrophages and T cells share the same I-A region (Farr et al., 1977, 1979; Ziegler and Unanue, 1980). These results suggest that the presentation of antigen to T cells generates a signal which stimulates macrophages to secrete IL-1. Interestingly however, addition of lymphokine-containing medium to macrophages resulted in relatively weak stimulation of IL-1 secretion (Farr et al., 1979). Thus if the signal which induces macrophage IL-1 secretion is a T cell-derived factor it must be rather unstable compared with other lymphokines or perhaps cell-cell contact

is required.

IL-1 is likely to play a central role in maintaining the proliferative response of T_H cells to antigen. It should be noted however, that lymphocyte clonal expansion and differentiation are the end result of a complex network of interactions involving various populations of cells and the soluble mediators they produce. For example, one important effect of IL-1 is to induce Lyt $1^+2^-3^-$ T cells to produce another major mediator known as interleukin 2 (IL-2) (reviewed by Howie and McBride, 1982a). IL-2 for its part causes continued proliferation of antigen- or mitogen-activated T cells (reviewed by Fathman et al., 1981). Responding T cells appear only to gain receptors for IL-1 and IL-2 following activation by antigen presented in conjunction with self Ia determinants (Smith and Ruscetti, 1981), once again emphasizing the central involvement of antigen presenting cells in initiating and maintaining T cell clonal expansion and differentiation.

Although IL-1 has been most studied in the context of its lymphostimulatory activity, it is evident that a similar, if not identical, factor may be involved in the stimulation of connective tissue cell proliferation (reviewed by Whicher and Chambers, 1984) induction of fever (Dinarello et al., 1974, 1982), stimulation of proteinase and prostaglandin production by connective tissue cells (Dayer et al., 1981), stimulation of the production of acute phase proteins (Wannenmacher et al.,

1975), bone resorption *in vitro* (Gowen *et al.*, 1983), B cell activation (Wood and Gaul, 1974) and the induction of neutrophilia (Merriman *et al.*, 1977). Several of these functions may be important in the development and maintainance of chronic inflammatory lesions (reviewed by Whicher and Chambers, 1984). In this connection it is interesting to note that both putative antigen presenting cells and T cells with the helper phenotype have been identified within various chronic inflammatory lesions (reviewed by Poulter, 1983) suggesting a role for these cells in the pathogenesis of such disease states.

3.5 Role of accessory cells in the generation of anti-tumour immune responses

Considering the vast amount of data that has been collected on accessory cell function it is perhaps rather surprising that so few of these studies examined the role of accessory cells in the generation of anti-tumour immune responses. Many factors contribute to the lack of progress in this area, not least of all the considerable difficulties associated with detecting specific immune responses to weak cellular antigens whose physiochemical nature is largely unknown and which may be unique to individual tumours. Moreover, there is a lack of simple, reproducible *in vitro* assay systems that are amenable to the manipulations necessary to enable to various cellular interactions involved in the

generation of anti-tumour immunity to be adequately studied. However, the few reports that are available do suggest a role for accessory cells in the immune response to tumour antigens.

In vivo, administration of microlitre quantities of anti-I-A antisera has been found to produce a marked enhancement of tumour growth in tumour-immune mice rechallenged with viable tumour cells (Perry et al., 1978). Studies in the ABA hapten system suggest that the primary effect of anti-I-A in vivo, may be at the level of the antigen presenting cell (Perry et al., 1980; Lowy et al., 1984). On the basis of this data, it seems likely that antigen presentation by I-A-positive accessory cells plays an important role in the development of anti-tumour responses in vivo. Supporting evidence comes from the finding that spleen cells removed from anti-I-A treated tumour-bearing mice contain a population of T_s cells which can suppress the anti-tumour immune response upon adoptive transfer (Perry et al., 1978). It is evident in other systems that the depletion of I-A-positive accessory cells can lead to the generation of antigen-specific T_s cells (Lowy et al., 1983, 1984).

Several early studies noted that populations of lymphoid cells cultured in vitro with allogeneic or xenogeneic fibroblasts (Ginsberg, 1970), allogeneic lymphocytes (Hayry and Defendi, 1970) or allogeneic or syngeneic tumour cells

(Ilfeld et al., 1973; Sharma and Terasaki, 1974; Small and Trainin, 1975) underwent a blastogenic response and acquired specific cytotoxic activity which could be readily demonstrated in vitro using the appropriate target cell. The cellular interactions involved in this response were not generally studied although Lonai and Feldmann(1971) did demonstrate that macrophages were required for the in vitro generation of cytotoxic lymphocytes against xenogeneic fibroblasts.

A role for macrophages in the in vitro generation of cytotoxic lymphocytes against syngeneic tumour cells was suggested by the studies of Treves et al. (1976). They observed that thioglycollate-elicited peritoneal macrophages pulsed with a cell-free preparation of syngeneic tumour could sensitize syngeneic spleen cells against the tumour. The effector cells were non-adherent to nylon wool, prevented tumour cell targets synthesizing DNA in vitro and inhibited tumour growth in vivo, most likely reflecting the presence of cytotoxic T cells. This cytotoxic response was generated by antigen-pulsed syngeneic macrophages but not allogeneic macrophages, fibroblasts or the antigen preparation by itself. The most likely interpretation of these studies is that macrophages can present tumour antigen for the primary generation of cytotoxic T cells.

A large number of studies have confirmed the requirement

for accessory cells in the generation of cytotoxic T cells not only against tumours but also against virally infected cells and modified autologous cells (Wagner et al., 1972; Koren and Hodes, 1977; Woodward and Daynes, 1979; Woodward et al., 1979; Friedman et al., 1979; Taniyama and Holden, 1979; Yamashita and Hamaoka, 1979; Pettinelli et al., 1980). However, the exact role of macrophages in this response, whether presenting antigen or merely supplying stimulatory factors, is by no means clear. Some reports demonstrated that the generation of primary cytotoxic T cells to tumour cells or allogeneic cells required the presence of Ia-positive macrophages although it did not seem to matter whether these were syngeneic or allogeneic to the responding lymphocytes (Yamashita and Hamaoka, 1979; Pettinelli et al., 1980). Similarly, although the in vitro generation of cytotoxic activity by T cells obtained from the lymph nodes draining a tumour mass required the presence of Ia-positive accessory cells the response did not need the addition of tumour antigen (Woodward and Daynes, 1979; Woodward et al., 1979). However, there has been at least one report which demonstrates an absolute requirement for syngeneic macrophages in the generation of cytotoxic T cells to syngeneic tumours (Taniyama and Holden, 1979). Together these results emphasize that the part played by macrophages and other accessory cells in this complex response is still far from clear.

3.6 Macrophage suppressor cell activity

Macrophages, particularly at high cell number, can exert potent inhibitory effects on various immune functions, including lymphocyte proliferation, the generation of cytotoxic T-lymphocytes, antibody responses, lymphokine production and NK cell activity (reviewed by Varesio, 1983). Both unstimulated macrophages (Fernbach *et al.*, 1976; Baird and Kaplan, 1977a,b; Varesio *et al.*, 1979a; Forni *et al.*, 1979) and cells activated *in vivo* (Kirchner *et al.*, 1975a; Kirchner, 1978; Yung and Cudkowicz, 1978; Farr *et al.*, 1979; Mehra *et al.*, 1979; Riglar and Cheers, 1980) or *in vitro* (Taramelli and Varesio, 1981) may possess this function and it has been suggested that this phenomenon represents a physiological mechanism that contributes to the homeostatic control of the immune system (Varesio *et al.*, 1979b; Varesio and Holden, 1980a; Herberman *et al.*, 1980).

Of particular interest are studies demonstrating the presence of suppressor macrophages in various tissues of tumour-bearing animals. Adler *et al.* (1971) observed a decrease in the induced proliferative response of spleen cells from mice bearing primary methylcholanthrene-induced tumours. Following tumour excision, the proliferative capability returned to normal levels. Gillette and Boone (1973, 1975) reported a similar decrease in the mitogenic response to PHA in mice bearing a variety of histologically distinct tumours. This

phenomenon was investigated further by Kirchner et al. (1974a,b) using spontaneously regressing Moloney murine sarcoma virus (MSV)-induced tumours. They found that the proliferative response to both ConA and PHA was normal during the early stages of tumour development, was maximally depressed at peak tumour size and rapidly returned to normal levels after tumour regression (Kirchner et al., 1974b; Weiland and Mussgay, 1977). Passage of hyporesponsive spleen cells over a rayon wool column or depletion of phagocytic cells by carbonyl iron treatment, completely restored responsiveness to PHA (Kirchner et al., 1974a,b, 1975a,b,). These results suggest that adherent and phagocytic spleen cells were responsible for the observed suppression and demonstrated that lymphocytes able to respond to the mitogens were present in the spleens of tumour-bearing animals. Additional evidence for the existence of suppressor cells was obtained in studies which showed that addition of spleen cells from tumour-bearing animals reduced the mitogen induced proliferative response of normal spleen cells. This inhibitory activity could be abrogated by removal of adherent, phagocytic and carrageenan-sensitive cells, but was unaffected by treatment with anti-Thyl or anti-Ig and complement and was not sensitive to X-irradiation, suggesting the involvement of a macrophage-like cell (Kirchner et al., 1974a, 1975a).

Suppressor macrophages have also been identified in the

spleens of mice bearing methylcholanthrene-induced tumours (Pope et al., 1976; Whitney et al., 1978) and spontaneous tumours (Whitney et al., 1978; Forni et al., 1979; Varesio et al., 1979a).

Much evidence in recent years has indicated that suppressor macrophages are also generated during human malignant disease. Peripheral blood leukocytes (PBL) from cancer patients frequently exhibit a decreased antigen- or mitogen-induced proliferative response (reviewed by Varesio, 1983). The study of Blomgren et al. (1976) was one of the first to suggest a role for suppressor macrophages in this phenomenon. They found that a considerable proportion of patients with cancer of the breast had a decreased lymphoproliferative response to PPD. However, following macrophage depletion the response generated returned to near normal levels.

Berlinger et al. (1976) demonstrated that passage of PBL over a Sephadex G10-column, a process known to deplete macrophages restored the depressed proliferative response of these cells in mixed lymphocyte cultures (Berlinger et al., 1976). Similar results were obtained by Berlinger and Good (1980) in a study of colon cancer patients.

Zembala et al. (1977) found that PBL from 15 out of 27 patients with advanced carcinomas of the lung or gastrointestinal

tract exhibited hyporesponsiveness to Con A or PHA. Adherent cells from such patients were usually able to exert suppressive activity on normal spleen cells, indicating the involvement of suppressor macrophages. However, in a few patients, T cells but not macrophages were shown to be responsible for the suppression. Similar results were obtained by Jerrells et al. (1978). Among 38 lung cancer patients, 15 had demonstrable suppressor cells. In experiments to characterize the nature of the suppressor cells, 5 out of 8 lung cancer patients were found to have adherent suppressor cells and the rest had nonadherent suppressor cells.

The effectiveness of suppressor macrophages in vivo remains the subject of considerable debate (reviewed by Varesio, 1983). Anaclerio et al. (1978) showed that spleen cells from mice bearing Lewis lung carcinoma had a depressed proliferative response to PHA and LPS and that this finding was attributable to the presence of suppressor macrophages. When spleen cells from tumour-bearing animals were mixed with normal spleen cells and injected into the footpad of allogeneic mice, a depression of the resultant graft-versus-host reaction was observed, suggesting that the suppressor macrophages were also functional in vivo. Moreoever, depletion of phagocytic cells by carbonyl iron treatment abrogated the depression of the in vivo response. In contrast, Forni et al. (1982) found that mice bearing a transplanted spontaneous

adenocarcinoma rejected allogeneic mastocytoma cells as well as normal mice. The rejection of the allograft in both normal and tumour-bearing animals was T cell dependent. However, this T cell function was clearly unaffected despite the presence of suppressor macrophages in the tumour-bearer which were capable of inhibiting in vitro lymphoproliferative responses and lymphokine production (Forni et al., 1979; Varesio et al., 1979a)..

Although this very limited data does not rule out an important role for suppressor macrophages in vivo, it seems unlikely that they induce a generalized anergy and their effects are probably much more restricted. It is possible that such cells exert their suppressive activity only when they are in close contact with responding lymphocytes. This situation is of course selected for in vitro studies. However, suppressor macrophages are not evenly distributed throughout tumour-bearing animals, e.g. they may be present in the spleen but absent from the lymph nodes (Gillette and Boone, 1975; Oehler et al., 1978) and it is therefore possible that only a limited number of lymphocytes will come into contact with suppressor macrophages in vivo.

4.0 ROLE OF MACROPHAGES IN THE EFFERENT ARM OF THE IMMUNE RESPONSE

4.1 Macrophage activation

Normal macrophages can phagocytose and kill pyogenic bacteria such as Staphyloccus aureus and Escherichia coli (van Zwet et al., 1975). There are however a number of diverse microbial pathogens that can survive inside normal macrophages and eventually kill them through unrestricted intracellular multiplication. Such organisms include the bacteria Brucella, Listeria, Mycobacteria and Salmonella (Mackaness, 1962, 1964, 1970), and the protozoa Leishmania (Farah et al., 1975; Behin et al., 1975), Toxoplasma (Remington et al., 1972) and Trypanosoma (Nogueira et al., 1977). Recently it was shown that the causative organism of Legionnaires disease, Legionella pneumophila could also survive and multiply inside normal macrophages (Nathan et al., 1980). Animals can however acquire impressive levels of immunity to infection with these intracellular parasites (Cohn, 1978). Such immunity was found not to be passively transferable by serum (Miki and Mackaness, 1964), but was related to the development of an enhanced microbicidal activity by the host macrophages (Mackaness, 1962, 1964). The term "activated macrophage" was introduced by Mackaness (1962) for the purpose of describing the intrinsic adaptive changes that enable macrophages to express enhanced anti-

microbial resistance. However, it is now evident that activated macrophages obtained from animals with acquired immunity to infection with intracellular parasites not only show enhanced antimicrobial activity against the infecting organism but are also able to non-specifically kill antigenically unrelated organisms (Mackaness, 1970), virally infected cells (Rodda and White, 1976) and transformed cells (Alexander and Evans, 1971).

Inflammatory stimuli induce dramatic changes in the morphological, biochemical and functional characteristics of macrophage populations. The response generated depends upon the nature of the inflammatory stimulus used. Some workers refer to these macrophages as "activated" (Cohn, 1978; Karnovsky and Lazdins, 1978), while others restrict the use of this term to describe cells with a proven ability to kill facultative intracellular parasites or cause extracellular lysis of tumour cells, preferring to use the terms "primed", "stimulated" or"angry" to refer to other states of altered activity (reviewed by Ogmundsdottir and Weir, 1980).

It is clear that within a population of macrophages elicited by inflammatory stimuli, not all macrophages are activated in the strict sense of having acquired the ability to kill intracellular parasites or tumour cells (Hibbs, 1974a;

Hibbs et al., 1977). Furthermore, different subpopulations may be described as "activated" depending upon which function is measured and how it is measured. For example, populations of cells with the ability to kill tumour targets can be physically separated from cells with enhanced bactericidal activity (Wing et al., 1977).

It is likely that there is more than one pathway which leads to macrophage "activation". Some agents e.g. lipopolysaccharide (LPS), pyran copolymer, poly I:C and certain lymphokines can "activate" populations of resident peritoneal cells in vitro to a state where they can kill tumour cell targets (Weinberg et al., 1978; Ogmundsdottir and Weir, 1980; Sone and Fidler, 1980; Taramelli and Varesio, 1981). These agents can act directly on macrophages and the process does not have to involve other cell types or their products. Macrophage "activation" in vivo may be more complex. Inflammatory stimuli induce an influx of poorly differentiated cells from the blood such that recently arrived cells quickly make up the majority of cells in a tissue site (Bursuker et al., 1982). In addition, the activating signals may be distinct from the eliciting agent and produced as the result of its interaction with host-elements, particularly lymphocytes and the complement system (reviewed by Ogmundsdottir and Weir, 1980).

Observations in several experimental systems suggest that activation of macrophages *in vivo* to a level where they can non-specifically kill tumour targets may occur via a sequential series of reactions (Hibbs *et al.*, 1977; Russell *et al.*, 1977; Ruco and Meltzer, 1978; Meltzer, 1981). Each stage of the activation process requires the similtaneous presence of appropriate activation signals and responsive macrophages.

It is likely that lymphokines play a central role in the activation process (reviewed by David *et al.*, 1983). Both sensitized lymphocytes (Koster and McGregor, 1970) and Ia-positive macrophages (reviewed by Poulter, 1983) have been identified within inflammatory sites. At least *in vitro*, the secretion of several T cell-derived mediators including macrophage inhibitory factor (MIF) (Ben-Sasson *et al.*, 1974; Wahl *et al.*, 1975; Ohishi and Onoue, 1975; Landolfo *et al.*, 1977), macrophage chemotactic factor (MCF) (Wahl *et al.*, 1975) and macrophage activating factor (MAF) (Farr *et al.*, 1979) has been shown to require antigen presentation by histocompatible Ia-positive accessory cells. Once secreted however, the various lymphocyte mediators act on all macrophages irrespective of their strain of origin or Ia content (Fidler, 1975; Riisgaard *et al.*, 1978; Farr *et al.*, 1979). Presumably within inflammatory sites Ia-positive macrophages can present antigen to immunocompetent

lymphocytes and thereby induce lymphokine secretion. These various lymphokines may then lead to local macrophage accumulation and activation.

The macrophages which accumulate at inflammatory sites have been shown to be 10-20 times more responsive to lymphokine-mediated activation than resident macrophages (Ruco and Meltzer, 1978). This increased responsiveness is not directly related to the poorly differentiated phenotype of inflammatory macrophages. For example, although peritoneal cells collected 6 hours after injection of irritant were highly responsive to lymphokine activation *in vitro*, peripheral blood monocytes from the same animals did not develop cytolytic activity even when exposed to very high concentrations of lymphokines for extended periods of time (Meltzer *et al.*, 1982). Moreover, macrophages derived from bone marrow precursors *in vitro* were refractory to lymphokine activation (Falk *et al.*, 1981). These studies were interpreted as demonstrating that the lymphokine reponsiveness of monocytes arriving at an inflammatory site is enhanced by exposure to local stimuli (Meltzer *et al.*, 1982).

Peritoneal exudate macrophages elicited by inflammatory stimuli rapidly develop strong cytolytic activity when exposed to high concentrations of lymphokines *in vitro* (Ruco and Meltzer, 1977; Meltzer *et al.*, 1980.) However, the

level of cytolytic activity generated decreased as the lymphokine was diluted. However if macrophages which had been exposed to low concentrations of lymphokines for 4-5 hours were then treated with nanogram quantities of LPS or high concentrations of lymphokines for 1 hour, strong cytolytic activity was generated (Ruco and Meltzer, 1978; Meltzer et al., 1980; Meltzer, 1981). The synergistic interaction between lymphokines and LPS was critically dependent upon treatment sequence. Cells treated similtaneously with lymphokine and LPS were strongly cytolytic; macrophages treated first with lymphokines for 5 hours and then with LPS for 1 hour were also cytolytic. In contrast cells treated with LPS for 1 hour and then exposed to lymphokines for 5 hours showed little or no cytolytic activity. However, the treatment with LPS did not preclude further responses to lymphokines since macrophages that were treated with LPS for 1 hour, lymphokines for 4 hours and again with LPS for 1 hour were cytolytic (Ruco and Meltzer, 1978; Meltzer et al., 1980; Meltzer, 1981).

On the basis of these results, the processes leading to the generation of cytolytically activated macrophages can be divided into three distinct phases. The first involves recruitment of immature macrophages to sites of inflammation and their conversion to lymphokine responsive cells by ill-

defined local factors. In the second phase these cells enter a "primed" non-cytolytic state in response to certain lymphokines. The third phase involves triggering of lymphokine-primed cells by other diverse signals to develop functional cytolytic activity.

Several different lymphokines may be involved in macrophage activation forming a network of interrelated signals (Gemsa et al., 1983) some of which may actually cause differentiation rather than activation (Onozaki et al., 1983). The precise functional capabilities of activated macrophages may depend on the nature of the lymphokines to which they were exposed (Gemsa et al., 1983). A better understanding of the effect of lymphokines on macrophages is sure to come from studies of well-defined factors derived from mitogen- or antigen-stimulated continuous T cell clones (Gemsa et al., 1983).

The simultaneous requirement for effective activation signals and competent macrophages at each stage in the activation process forms the basis of the control mechanism regulatingmacrophage effector function. Both the responsiveness of macrophages to activation signals and the final cytolytic state are short-lived functions which are rapidly lost with time and cannot subsequently be regained (Ruco and Meltzer,

1977; Meltzer et al., 1980; Meltzer et al., 1982; Meltzer, 1981). Thus activated macrophages will only persist if activation stimuli and responsive macrophages are constantly replaced.

4.2 Macrophage cytotoxic activity

Macrophages may exhibit a spectrum of cytotoxic reactions against tumour cells in vitro ranging from transient inhibition of cell growth to irreversible lysis (Meltzer et al., 1975; Keller, 1976; Kaplan et al., 1977). The final outcome of the interaction between macrophages and tumour cells in vitro presumably depends on the state of activation of the macrophage population, the sensitivity of the tumour targets and the conditions employed in the assay system. It is clear that within a population of activated macrophages not all cells are able to lyse tumour targets (Hibbs, 1974a; Hibbs et al., 1977). Moreover, the expression of cytotoxic activity is not a property of cells sharing particular physical, morphological or biochemical characteristics but varies with the system under investigation. In some systems small cells appear to be particularly cytoxic (Ruco and Meltzer, 1978), while in others, large cells are more effective (Lee and Berry, 1977; Lee et al., 1981).

The cytotoxic activity of activated macrophages is

immunologically non-specific and such cells efficiently kill a variety of syngeneic, allogeneic and xenogeneic neoplastic targets while leaving non-neoplastic cells unharmed (reviewed by Fidler and Raz, 1981). The mechanism by which activated macrophages descriminate between neoplastic and non-neoplastic cells is not clear. Macrophage-mediated cytotoxicity, at least in vitro appears to occur independently of tumour cell immunogenicity, drug sensitivity, invasiveness and metastatic potential (Fidler and Raz, 1981; Fidler and Poste, 1982; Loveless and Heppner, 1983). For example, B16 melanoma variant cell lines of high or low metastatic potential which were either susceptible or resistant to syngeneic T cell-mediated lysis were all equally susceptible to lysis in vitro by lymphokine-activated macrophages (Fidler and Raz, 1981). Similarly cell lines derived from the UV-2237 fibrosarcoma which differed greatly in invasive and metastatic potential in vivo (Fidler and Cifone, 1979), as well as immunogenicity and susceptibility to NK cell-mediated lysis (Hanna and Fidler, 1980) were all killed by activated macrophages in vitro (Fidler and Poste, 1982). Attempts to isolate a tumour cell variant line resistant to macrophage cytolysis by repeated selection of the small percentage of B16 melanoma or UV-2237 fibrosarcoma target cells which survived exposure to activated macrophages in vitro proved unsuccessful (Fidler and Poste, 1982). Cell lines resistant

to cytolysis by T cells (B16) or NK cells (UV-2237) were readily obtained by selection with the appropriate effector cells. However, all these variants were still susceptible to macrophage-mediated cytolysis. These studies suggest that cytolytic macrophages recognize some tumour cell characteristic which is intimately linked to the malignant phenotype but distinct from that recognized by T cells or NK cells.

Macrophage mediated cytolysis has generally been shown to be contact dependent, although phagocytosis does not seem to be involved (Evans and Alexander, 1976; Hibbs et al., 1978). Macrophages activated in vivo by BCG infection or pyran copolymer (Marino and Adams, 1980a, 1980b; Marino et al., 1981) or in vitro by lymphokines (Piessens, 1978; Marino and Adams, 1980a) exhibited strong high-level selective binding to neoplastic target cells. In contrast, the binding between non-activated macrophages and neoplastic target cells or between activated macrophages and a variety of non-neoplastic target cells was weak, low-level and mostly non-specific (Piessens, 1978; Marino and Adams, 1980a, 1980b; Marino et al., 1981). Examination of the interface between activated macrophages and attached targets by transmission electron microscopy revealed a zone of very close apposition between the two cells (Marino and Adams, 1980b; Adams et al., 1982). Indeed Hibbs (1974b) went as far as to suggest that activated macrophages

actually fuse with the target cells.

Target cell binding although necessary, is not in itself sufficient for macrophage mediated cytotoxicity. Macrophages primed *in vivo* by BCG or *in vitro* by lymphokines bound neoplastic target cells efficiently but did not lyse them until the macrophages were pulsed with traces of LPS (Adams and Marino, 1981; Adams *et al.*, 1982). Similarly, activated macrophages cultured overnight lost the capacity to lyse tumour cells although they were still able to efficiently bind the target cells (Adams and Marino, 1981).

Activated macrophages are very active secretory cells and synthesize and secret many substances that are not produced by resident macrophages or are synthesized without being secreted (reviewed by Davies and Bonney, 1979; Nathan *et al.*, 1980).

Several substances secreted by activated macrophages have been proposed as mediators of their cytotoxic activity.

(a) Lysosomal enzymes

Activated macrophages secrete large amounts of lysosomal enzymes (reviewed by Davies and Allison, 1976). Hibbs (1974b) observed that lysosomal material was transferred from activated macrophages to attached tumour cell targets and proposed that secreted lysosomal enzymes were producing the resultant cytolytic effect, probably through their hydrolytic activity. However, the role of lysosomal enzymes in target cell killing has been questioned by the finding that the addition of even quite high concentrations of lysosomal enzymes to culture medium usually enhanced rather than inhibited tumour cell growth (Vischer et al., 1976). Clearly if lysosomal enzymes do mediate macrophage cytotoxic activity it must be by a mechanism other than a direct attack on the exterior surface of the target cell.

(b) Oxygen metabolites

Many stimuli induce activated macrophages to secrete reactive oxygen metabolites (Johnston et al., 1978). Several lines of evidence suggest that these products play an important part in the killing of tumour cell targets. Firstly, target cells are susceptible to lysis by oxygen metabolites in the amounts released from activated macrophages in response to phorbol myristate acetate (PMA) (Nathan et al., 1979a).

Secondly, deprivation of either oxygen or glucose prevents PMA-induced oxygen metabolite release from activated macrophages and greatly reduces target cell killing (Nathan et al., 1979b; Nathan and Cohn, 1980). Finally, exogenous scavengers of oxygen metabolites such as catalase or thioglycollate broth, abolish PMA induced target cell killing by activated macrophages (Nathan et al., 1979b; Nathan and Cohn, 1980).

(c) Neutral proteinases

It is a frequent finding that culture supernatants conditioned by activated macrophages incubated alone or with tumour cells, were not cytotoxic to tumour targets in vitro (reviewed by Alexander, 1976; Evans and Alexander, 1976). Similarly if activated macrophages and target cells were separated by a porous filter, no cytotoxicity resulted if serum was present in the culture medium (Marino and Adams, 1980b). In contrast, if the same experiment was performed in the absence of serum, significant cytotoxic activity was evident (Adams and Marino, 1981). These results strongly suggest that activated macrophages do secrete a soluble cytoxic product, but that this substance is effectively inhibited by serum components.

Further studies using additional inhibitors (Adams, 1980; Adams et al., 1980; Johnson et al., 1981) and various

biochemical techniques (Adams et al.,1980) identified the cytotoxic principle present in culture supernants conditioned by activated macrophages as a neutral serine proteinase with a molecular weight of approximately 40,000 daltons. However, it should be noted that inhibitors of neutral proteinase activity may exert numerous effects including the suppression of reactive oxygen metabolite release by activated macrophages (Kitagawa et al., 1980).

(d) Complement component C3a

Activated macrophages secrete neutral proteinases and complement component C3 (reviewed by Nathan et al., 1980). Neutral proteinases can cleave C3 to give C3a and C3b fragments. It has been suggested that C3a is cytotoxic for tumour target cells (Ferluga et al., 1976). However recent work by Goodman et al. (1980) has cast serious doubt on the cytotoxic capacity of C3a and clearly much more work needs to be carried out to clarify the importance of C3a in target cell killing.

(e) Arginase

Another recent suggestion is that arginase is responsible for the cytolytic capacity of activated macrophages (Currie, 1978). Arginase was shown to be released by macrophages activated in vitro by zymosan or LPS but not by normal macro-

phages (Currie, 1978). It is presumed that arginase exerts its cytolytic effect by preventing the target cell obtaining sufficient arginine (Currie, 1978).

(f) Thymidine

Stadecker et al., (1977) suggested that the nucleoside thymidine is responsible for target cell killing. Thymidine is synthesized and secreted by activated macrophages (Stadecker et al., 1977) but few target cells are likely to be susceptible to lysis at the concentrations of thymidine attained.

In summary, it appears that lysis of target cells by activated macrophages occurs in at least three stages. In the first stage, activated macrophages bind to target cells creating a zone contact between the two cells and triggering metabolic events within the macrophage. In the second stage macrophages secrete lytic effector substances into the zone of contact. It is likely that several such substances are involved and the relative importance of each may vary depending on the target cells or environmental conditions. The contact zone presumably acts to concentrate the lytic substances and protect them from inhibitors present in the extracellular compartment. Finally, the third stage involves target cell injury and eventual loss of viability.

4.3 Macrophage-induced stimulation of tumour cell proliferation

It is apparent that under some conditions, immune reactions may stimulate cell proliferation rather than inhibit it (Prehn, 1976). Normal peritoneal macrophages have been shown to enhance the growth of lymphoid tumour cells which by themselves grew poorly *in vitro* (Namba and Hanaoka, 1972; Nathan and Terry, 1975; Hewlett *et al.*, 1977). Similarly, Olivotto and Bomford (1974) reported that cultured mouse peritoneal macrophages stimulated ^{3}H-thymidine uptake by tumour cells. Moreover, enhancement of tumour cell proliferation is not limited only to resident macrophages. Krahenbuhl *et al.* (1976) showed that peritoneal macrophages from animals injected with *Corynebacterium parvum* or killed *Toxoplasma gondii* greatly enhanced ^{3}H-thymidine uptake by L-929 cells at low effector to target cell ratios. Similar results were reported for proteose-peptone activated macrophages (Keller, 1973).

The *in vivo* significance of the tumour growth promoting activity of macrophages remains to be elucidated. Hewlett *et al.* (1977) suggested that macrophages enhance tumour cell proliferation *in vitro* simply by improving culture conditions. It is of course unlikely that an equivalent mechanism would be of importance *in vivo*. However, Prehn (1977) proposed that immunostimulation of tumour cell proliferation plays an

important part in the early stages of tumour development *in vivo*. In this connection it is interesting to note that in one study treatment of mice with the anti-macrophage agent silica was reported to inhibit the growth of subcutaneously implanted Lewis lung carcinoma cells (Sadler *et al.*, 1977).

5.0 TUMOUR-ASSOCIATED MACROPHAGES

5.1 Accumulation of macrophages in tumours

The accumulation of macrophages in tumours appears to be a well-regulated process. It is for instance generally accepted that although different tumours may contain different numbers of macrophages, once established, the macrophage content of a given tumour remains fairly constant during passage from one syngeneic host to another (Evans, 1972; Pross and Kerbel, 1976; McBride *et al.*, 1982). Thus while primary chemically induced tumours may contain a high percentage of macrophages, this number often decreases sharply during early passage, eventually leveling off at a figure which may be maintained through many subsequent *in vivo* passages (Evans, 1972; Pross and Kerbel, 1976; McBride *et al.*, 1982). In this connection, it is interesting to note that the immunogenicity of chemically induced tumours may also decrease during early passage (Prehn and Main, 1957; Globerson and Feldman, 1964; Menard *et al.*, 1973). The most likely explanation of these results is that repeated transplantation selects tumour cells particularly well adapted to growth under the set conditions that transplantation imposes and that such cells elicite, by some well regulated process, a characteristic host cell infiltrate (McBride *et al.*, 1982).

The host cell origin of tumour-associated macrophages has been demonstrated in several studies. Tumour cell suspensions depleted of macrophages prior to inoculation into syngeneic hosts by treatment with anti-macrophage serum and complement (Evans, 1972) or by long term *in vitro* culture (Evans, 1972; Kerbel and Pross, 1976; Moore and Moore, 1977a) still developed a characteristic macrophage infiltrate, suggesting that tumour-associated macrophages were not derived from cells included in the tumour inoculum. Confirmation of the host origin of tumour-associated macrophages was obtained by growing parental strain tumours in F_1 hybrid hosts (Kerbel *et al.*, 1975; Kerbel, 1976; Kerbel and Pross, 1976; Pross and Kerbel, 1976). For example, CBA ($H\text{-}2^k$) methylcholanthrene-induced fibrosarcoma cells were injected into (C3H x DBA/2)F_1 ($H\text{-}2^k$ x $H\text{-}2^d$) recipients. Host cells infiltrating the tumour expressed $H\text{-}2^k$ and $H\text{-}2^d$ antigens since histocompatibility antigens are codominant, and were identified with anti-$H\text{-}2^d$ antisera. Tumour cells on the other hand expressed only $H\text{-}2^k$ antigens and did not react with anti-$H\text{-}2^d$ antisera (Kerbel and Pross, 1976).

The current dogma concerning the origin of macrophages in normal tissues or inflammatory sites suggests that these cells are derived mainly from blood monocytes, although in some circumstances, *in situ* macrophage proliferation may

also occur (section 2.2). The accumulation of macrophages within tumours presumably occurs by the same mechanism. Influx of monocytes into a tumour site undoubtably occurs (DeLusto and Haskill, 1978; Talmadge et al., 1981). However, identification of recently arrived monocytes in tumour cell suspensions by peroxidase staining, revealed very little staining that could not be accounted for by neutrophil contamination (Evans, 1982). This suggests a very rapid differentiation of tumour-infiltrating monocytes into macrophages (Kaizer and Lala, 1977). The extent of in situ macrophage proliferation is much more uncertain. Following i.v. injection of ^{3}H-thymidine, labelled macrophages were found in situ (Key and Haskill, 1981). In addition, in the presence of CSF, tumour-associated macrophages may form colonies in vitro (Stevens et al., 1978). However, preliminary data on labelling indices following i.v. injection of ^{3}H-thymidine were shown to be too low to account for the observed increase in macrophage number as the tumour grows (Evans, 1982). In addition, tumour cell suspensions pulsed with ^{3}H-thymidine in vitro showed a similar low macrophage labelling index (Evans, 1982). In conclusion, it seems likely that most tumour associated macrophages are derived from an influx of blood monocytes into the tumour site although some in situ macrophage proliferation may also occur. The relative contribution of each of these mechanisms

presumably varies between different tumours. Some tumours have been shown to produce CSF (Milas et al., 1984) and with these, local macrophage proliferation may contribute significantly to the total macrophage content.

Numerous authors have investigated the degree of macrophage infiltration of tumours as a function of time after injection of tumour cells. It appears from these studies that the kinetics of macrophage infiltration may also vary from one tumour to another. In some, the percentage of macrophages peaked early in growth before declining to a level which was maintained throughout the remaining period of growth (Moore and Moore, 1977a; Evans and Eidlen, 1981). With other tumours, the same percentage of macrophages was found throughout the period of growth (Evans, 1972). In contrast, the macrophage content of several sarcomas and carcinomas has been shown to remain relatively constant until a critical tumour size is reached, after which time it decreased substantially, perhaps reflecting diminished infiltration of blood monocytes (Haskill et al., 1975a; Moore and Moore, 1977a; Normann and Cornelius, 1978; Evans and Eidlen, 1981).

Several possible mechanisms may account for the accumulation of macrophages in tumours.

(a) Mediators of acute inflammation

Evans (1972) reported no apparent correlation between the macrophage content of tumours and the degree of necrosis or neutrophil infiltration. In addition the anti-inflammatory drug indomethacin failed to affect the macrophage content of a variety of rat sarcomas and carcinomas (Moore and Moore, 1977b). Similarly, the non-steroidal anti-inflammatory agent flurbiprofen which effectively inhibits prostaglandin synthesis did not alter either the migration of labelled monocytes into rat sarcomas or the number of macrophages found within these tumours (Eccles, 1982). Thus, on the basis of this evidence, it seems unlikely that macrophages are attracted to a tumour site by mediators of acute inflammation.

(b) Immune mechanisms

It has been reported that the levels of macrophages associated with tumours appears to be related to the immunogenicity of tumour cells, such that highly immunogenic tumours have a relatively high macrophage content, and weakly immunogenic tumours a low content (Evans, 1972; Eccles and Alexander, 1974; Moore and Moore, 1977a; Morantz et al., 1979). Such correlations were most clearly seen when tumours of similar aetiology,histology and host origin were compared (Eccles and Alexander, 1974). Of particular interest in this respect

are pairs of tumours where the macrophage content of an immunogenic line and its less immunogenic derivative were compared. For example, the macrophage content of the strongly immunogenic benzo(a)pyrene-induced sarcoma FS6 was around 44% while the weakly immunogenic mFS6 subline contained only 19% (Mantovani, 1978). Similar results were obtained using the L5718Y Eb and ESb lymphomas (Schirrmacher et al., 1979).

The correlation between tumour immunogenicity and macrophage content strongly suggests that macrophage accumulation is sustained by stimuli which are immunological in nature. This hypothesis is supported by studies which demonstrated a significant reduction in the macrophage content of various sarcomas and carcinomas grown in T lymphocyte depleted (Eccles and Alexander, 1974; Alexander et al., 1976; Parthenais and Haskill, 1979) or nude (Stutman, 1975; Eccles et al., 1979) animals. In addition, administration of Cyclosporin A, a potent immunosuppressive agent with selective inhibitory effects on T lymphocytes (White et al., 1979) decreased both the migration of labelled monocytes into rat sarcomas and the number of macrophages found within these tumours (Eccles, 1982).

However, the suggested involvement of specific anti-tumour immune responses in the accumulation of macrophages in tumours

is not supported by all studies. Evans and Lawler (1980) for instance, found no correlation between immunogenicity and macrophage content in an extensive study of 33 primary methylcholanthrene induced murine sarcomas. In addition, Szymaniec and James, (1976) found that the levels of tumour-associated macrophages in a mouse sarcoma were unaffected by transplantation into thymectomized animals. Similar findings have recently been reported for other methylcholanthrene-induced murine sarcomas transplanted into T-lymphocyte depleted (Evans and Lawler, 1980; Evans and Eidlen, 1981) or nude (Evans, 1982) mice.

In view of the contradictory nature of some of these studies it is probably best to adopt a cautious approach. It appears that at least for some tumours, there is a good correlation between immunogenicity and macrophage content. However this is by no means always the case, and for other tumours, different mechanisms may be more important. Many more studies must be performed before it can be ascertained whether the relationship between tumour immunogenicity and macrophage content applies to most, or to only a few tumours.

(c) Tumour-derived chemotactic factors

The accumulation of leukocytes at inflammatory foci is

part of the host defense response to many noxious agents. One of the mechanisms which leads to such localization is the elaboration of molecules chemotactic for leukocytes that diffuse out from the site of release. Leucocytes then migrate up the concentration gradient to form the inflammatory focus. Neutrophils, basophils, eosinophils and macrophages all respond to various chemotactic stimuli. Factors which preferentially attract a particular type of leukocyte have been described. Thus the nature of chemotactic factors may determine the cellular composition of inflammatory foci.

There have been a few reports indicating that tumour cells may elaborate factors that are chemotactic for macrophages. Meltzer et al. (1977) found that culture supernatants from 4 different murine methylcholanthrene-induced fibrosarcomas contained factors that were chemotactic for resident and BCG-activated peritoneal macrophages but not for peritoneal granulocytes. Activated peritoneal macrophages were more responsive to the tumour-derived chemotactic factors than were resident peritoneal macrophages. Minimal levels of chemotactic activity were found in supernatants from syngeneic mouse embryo fibroblast cultures. Similarly, in a recent extensive study Bottazzi et al. (1983) found chemotactic activity in the culture supernatants of most

murine and human sarcomas and carcinomas irrespective of their histology or origin (chemically induced or spontaneous). Leukaemias and lymphomas on the other hand were either inactive or elaborated a low chemotactic activity. The elaboration of chemotactic factors by human tumour cells *in vitro* was confirmed by Peri *et al.* (1982) who demonstrated that culture supernatants from the human sarcoma cell line 8387 and the human melanomas 1080/2 and SKBR-3 were chemotactic for human monocytes whereas the K562, Raji and CEM lymphoma lines produced little chemotactic activity.

The physiochemical nature of tumour derived chemotactic factors has been examined in at least two systems. Meltzer *et al.* (1977) found that the chemotactic activity present in the culture supernatant of a murine fibrosarcoma line eluted from Sephadex G100 in the 15,000 dalton molecular weight region. Similarly, preliminary characterization of the chemotactic factor elaborated by the human sarcoma line 8387 *in vitro* revealed that the activity was non-dialysible, heat-resistant, susceptible to proteolytic enzymes but unaffected by DNase or RNase and eluted from Sephadex G75 in the cytochrome C region, corresponding to a molecular weight of round 15,000 daltons (Bottazzi *et al.*, 1983). Thus it seems that the chemotactic factors elaborated by murine and human tumour cells *in vitro* are fairly similar with respect

to molecular weight and may possibly be related. Indeed, chemotactic factors produced by murine cells were found to be active on human monocytes (Bottazzi *et al.*, 1983).

The elaboration of chemotactic factors by tumour cells has only been demonstrated *in vitro*. However, Bottazi *et al.* (1983) found a significant although far from absolute relationship between the macrophage content of 11 solid murine tumours and the ability of the tumour cells to produce chemotactic factors *in vitro*, suggesting that such a mechanism may also be important *in vivo*. Of course *in vivo*, the interaction of tumour cells with various host elements may result in the production of additional chemotactic factors. However, the macrophage content of primary and transplanted tumours was the same whether they were grown in normal or C5 deficient mice, indicating that C5a-derived chemotactic factor was not involved (Evans, 1980). The possibility that soluble complexes of tumour antigen and antibody may attract macrophages to a tumour site has been proposed (Evans, 1977) but remains speculative.

(d) Tumour-derived chemokinetic factors

Several authors have described tumour cell-derived factors which can act up upon macrophages *in vitro* increasing their rate of random translational movement (chemokinesis).

For example, Meltzer et al. (1975) using time lapse cinemicrographic analysis observed that the rate of translational movement of BCG-activated murine peritoneal macrophages cocultured with neoplastic cells was four times that observed if the macrophages were cocultured with non-neoplastic cells derived from the same cloned embryo cell line. The translational movement of resident peritoneal macrophages on the other hand was less than that of activated macrophages and was unaltered by the addition of neoplastic or non-neoplastic cells. Snodgrass et al. (1976, 1977) obtained similar results for resident and pyran copolymer activated murine peritoneal macrophages cocultured with Lewis lung carcinoma cells. In their studies, activated macrophages alone moved almost three times faster than resident macrophages. The rate of translational movement of resident macrophages was unaltered by coculture with neoplastic Lewis lung carcinoma cells or with non-neoplastic mouse embryo fibroblasts. Activated macrophages on the other hand moved three times faster when coincubated with, but not actually in contact with, carcinoma cells. However, when such a macrophage came into contact with a tumour cell, its rate of lateral movement slowed to one comparible with activated macrophages cultured alone. In contrast, activated macrophages cocultured with non-neoplastic mouse embryo fibroblasts showed no alteration in their rate of translational movement.

These results suggest that neoplastic cells elaborate chemokinetic factors which act to increase the rate of translational movement of activated macrophages. Indeed, Snodgrass et al. (1976; 1978) and Schuller et al.(1978) have shown that addition of cell free culture supernatant from Lewis lung carcinoma cells greatly enhanced the translational movement of pyran copolymer-activated murine peritoneal macrophages while supernatant from primary mouse embryo fibroblast cultures had no effect. The rate of translational movement of resident peritoneal macrophages was either unaltered (Snodgrass et al., 1976; 1978) or inhibited (Schuller et al., 1978) by addition of supernatant from Lewis lung carcinoma cell cultures.

In a recent study, Lane et al. (1982) found that culture supernatants from six different syngeneic and allogeneic mouse tumour cell lines all increased the rate of translational movement of Corynebacterium parvum-activated murine peritoneal macrophages while supernatants from normal mouse embryo fibroblast cultures had no effect. The chemokinetically active component of Lewis lung carcinoma conditioned medium was partially characterized and found to be a trypsin sensitive, heat stable, high molecular weight (300,000-400,000 dalton) factor that exhibited no chemotactic activity. The factor increased the rate of translational movement of C.parvum-and pyran copolymer-actived macrophages but had no effect on oyster

glycogen-or thioglycollate-elicited macrophages.

Reports on the molecular size of chemokinetic factors isolated from other tumours have been rather vague. Usually they were described as non-dialyzable indicating a molecular weight of greater than 12,000 daltons (Nelson and Nelson, 1978). Since all of the tumour-derived chemokinetic factors which have been described have a molecular weight above 12,000 daltons it is possible that all these molecules are related.

Several factors with chemokinetic activity have been isolated from other sources. Aaskov and Anthony (1976) described a high molecular weight (> 150,000 daltons) factor in culture supernatants of BCG-stimulated human peripheral blood lymphocytes which enhanced the movement of normal mouse splenic macrophages in the MIF assay but had no chemotactic activity. A similar factor was found in supernatants of PPD stimulated lymphocytes (Weisbart et al., 1974). This factor was heat stable, had an electrophoretic mobility in the gamma-globulin region and increased the random movement of human buffy coat leukocytes from a well cut in agarose gel. Thus it appears that the physiochemical features of tumour-derived chemokinetic factors are not unusual when compared with factors from other sources. At present however, the precise nature of chemokinetic factors remains to be elucidated. In this connection, it is interesting to note that cell coat glycocalyx glycoproteins have

a molecular weight range of 200,000 to 500,000 daltons (Codington et al., 1972; Slayter and Codington, 1973) which is fairly similar to that suggested for chemokinetic factors.

In summary, it appears that at least in vitro, neoplastic cells may elaborate factors which can enhance the random movement of activated macrophages while having little or no effect on non-activated macrophages. If these factors are produced in vivo and have similar effects on monocytes, then they could serve to specifically increase the rate of migration of activated cells out of the vascular system in response to an appropriate stimulus, while having little or no effect on non-activated cells. In this way these factors could influence not only the absolute number of macrophages in a tumour, but also their activity.

(e) Depression of macrophage chemotactic and inflammatory responses by tumour-derived factors

Patients with advanced malignant disease are often unable to express delayed type hypersensitivity (DTH) reactions to antigens with which they had previously been sensitized (Southam, 1968) or produce normal cellular exudates in response to mild skin abrasions (Dizon and Southam, 1963) or inflammatory stimuli (Johnson et al., 1971). Equivalent results have been obtained in animal studies using both spontaneously arising (Normann et al., 1979a; Cianciolo et al., 1980) and transplanted (Bernstein et al., 1972; Normann and Sorkin, 1976; Snyderman

et al., 1976; Normann and Cornelius, 1978; Normann et al., 1979b) tumours.

In most instances the anergy associated with tumour burden appears to be the result of a macrophage rather than a lymphocyte defect. Lymphocytes from tumour-bearing animals usually manifest normal in vitro responses to both antigens and mitogens (reviewed by Cianciolo and Snyderman, 1982). In contrast, several macrophage functions have been shown to be compromised in tumour-bearing animals or patients with advanced malignant disease (reviewed by Cianciolo and Snyderman, 1983). Of particular importance is the finding that macrophage chemotactic responsiveness in vitro is greatly reduced (Hausman et al., 1973, 1975; Boetcher and Leonard, 1974; Pike and Snyderman, 1976; Rubin et al., 1976; Snyderman et al., 1977; Snyderman et al., 1978). Such inhibition only becomes evident late in tumour progression and rapidly disappears if the tumour is excised (reviewed by Cianciolo and Snyderman, 1982). The most likely interpretation of these results is that tumours elaborate factors which inhibit macrophage chemotactic responsiveness.

Cell free homogenates of transplanted tumours (Pike and Snyderman, 1976; Snyderman and Pike, 1976; Cheung et al., 1979), tumour ascitic fluid (Normann, 1978), plasma or urine from tumour-bearing animals (Snyderman and Cianciolo, 1979) and

tumour cell culture supernatants (Normann and Sorkin, 1977; Normann and Cornelius, 1982) have all been shown to inhibit macrophage chemotaxis *in vitro* and/or depress macrophage accumulation at inflammatory sites *in vivo*. Similarly, Cianciolo *et al*. (1981) found inhibitory activity in all of 17 malignant effusions from patients with various types of neoplasms, whereas effusions from 17 patients with non-malignant diseases possessed no significant inhibitory activity.

The physiochemical nature of tumour-derived anti-chemotactic anti-inflammatory factors has been investigated in several systems. Pike and Snyderman (1976) identified an anti-inflammatory factor with a molecular weight of between 6,000-10,000 daltons in sonicates of 4 histologically distinct tumours. Cianciolo *et al*. (1980) found a similar factor in spontaneously arising murine carcinomas. Nelson and Nelson (1978, 1980) reported that murine tumour cell culture supernatants contained both low (< 10,000 daltons) and high (> 10,000 daltons) molecular weight factors which could inhibit the early phase of a prolonged DTH reaction *in vivo* and macrophage migration and motility *in vitro*. Similarly, Norman and Cornelius (1982) found that the P815 mastocytoma and a murine methylcholanthrene-induced fibrosarcoma both produced at least two soluble anti-inflammatory factors that inhibited macrophage accumulation *in vivo* when injected into syngeneic recipients. One factor was a low molecular weight peptide (< 1,000 daltons)

while the other had a molecular weight of between 30,000-100,000 daltons. Cianciolo et al. (1981) found that the anti-chemotactic factor present in human malignant effusions was a polypeptide of molecular weight 15,000-70,000 daltons. Interestingly this factor also reacted with monoclonal antibodies against the P_{15} (E) structural protein of retroviruses.

The mode of action of these tumour-derived anti-chemotactic anti-inflammatory factors has received little attention. Normann and Sorkin (1977) however found that culture supernatants from two rat tumour cell lines inhibited both macrophage accumulation in vivo in response to i.p. injection of peptone and the chemotactic responsiveness of peptone elicited-peritoneal macrophages in vitro if mixed with the chemotactic agent or cells. If the responding macrophages were incubated with culture supernatnants for a short period of time and then washed their chemotactic responsiveness was also greatly reduced. The supernatants had no effect on macrophage viability and repetitive absorptions by macrophages, depleted culture supernatants of their capacity to inhibit the chemotactic responsiveness of fresh macrophages. Other groups obtained similar results using sonicated murine tumour cell dialysates (Cianciolo and Snyderman, 1982) and human malignant effusions (Cianciolo et al., 1981). These results suggest that tumour-derived anti-chemotactic factors act by binding to the macrophage cell surface. The events

which occur following this interaction remain to be elucidated.

The part played by tumour-derived anti-chemotactic/anti-inflammatory factors in determining the macrophage content of tumours is difficult to evaluate. Although the effects of these inhibitors only become systemically evident late in tumour growth, it is possible that within the locality of the tumour mass functionally significant levels of inhibitors may be found at a much earlier stage. However no study to date has attempted to correlate the production of anti-inflammatory factors by tumour cells with the macrophage content of the tumour. Moreover, no one has yet examined the production of both chemotactic and anti-chemotactic factors within the same tumour system. If indeed certain tumours do produce both chemotactic and anti-chemotactic factors the two acting together could perhaps provide a rate limiting mechanism which determines the macrophage content of the tumour.

5.2 Functional capabilities of tumour-associated macrophages

As previously discussed in section 2, macrophages have been shown to exhibit considerable functional heterogeneity. Thus, in order to define the biological significance of tumour-associated macrophages, it is necessary to detail their functional capabilities. The standard approach used is to isolate macrophages from enzymatically-disaggregated tumour cell suspensions usually by virtue of their ability to adhere tightly to glass

or plastic surfaces, and then examine the functional capabilities of such cells in well-defined *in vitro* assay systems. Using this approach, tumour-associated macrophages have been shown to perform several functions which could have a bearing on tumour growth if they also occurred *in situ*.

(a) Cytotoxic activity

The frequent presence of macrophages in human and animal tumours, taken together with the observation that macrophages from other sites, activated *in vivo* or *in vitro* by a variety of agents, were able to kill phenotypically diverse tumour cells (see Section 4.2)prompted many groups to investigate the cytotoxic activity of tumour-associated macrophages. It has since been demonstrated that macrophages isolated from a wide variety of human and animal tumours, differing greatly in their origin and histology, may exhibit a spectrum of cytotoxic reactions *in vitro* which may be specific or non-specific and range from transient, reversible growth inhibition to irreversible lysis (Evans, 1973; Evans, 1976; Holden *et al.*, 1976; Russell and McIntosh, 1977; Korn *et al.*, 1978; Mantovani, 1978; Loveless and Heppner, 1983). The mechanisms behind such cytotoxicity are unknown but are unlikely to differ from those proposed for other populations of tumouricidal macrophages (Section 4.2).

In addition to direct cytotoxicity, tumour-associated

macrophages may also destroy tumour cell targets *in vitro* by ADCC (Haskill and Fett, 1976). In this connection it is interesting to note, that several studies have demonstrated the presence of immunoglobulins of various classes either bound to tumour cells (Witz, 1977; Koneval *et al.*, 1977; James *et al.*, 1978, 1979) or tumour-associated macrophages (Lewis *et al.*, 1976; Wood *et al.*, 1979) *in situ*. In addition, small numbers of plasma cells are found associated with many tumours (Ioachim *et al.*, 1976; Schoorl *et al.*, 1976; Howie *et al.*, 1982).

(b) Stimulation of tumour cell proliferation

It is apparent, that under some conditions, immune reactions may stimulate cellular proliferation rather than inhibit it. (Section 4.3) A number of investigators have demonstrated that tumour-associated macrophages may stimulate neoplastic cell proliferation *in vitro* (Evans, 1977, 1978; Mantovani, 1978; Salmon and Hamburger, 1978), or tumour growth in a Winn assay (Evans, 1978). Evans (1976) demonstrated that both neoplastic and non-neoplastic cells were stimulated to divide by culture medium conditioned by macrophages from solid murine tumours. Membrane filtration studies indicated that growth stimulation of SL-2 lymphoma cells by culture supernatant conditioned by macrophages isolated from the FS6 fribrosarcoma was mediated by a low molecular weight ($\leq$ 10,000 daltons) factor (Evans, 1979).

Salmon and Hamburger (1978) reported that a similar growth promoting factor was produced by macrophages from human malignant effusions. In some circumstances, factors produced by macrophages may replace substances such as 2-mercaptoethanol(2ME), which are frequently added to culture media to support the growth and proliferation of lymphoid cells. For example, the L5178Y lymphoma failed to grow in culture unless the culture medium contained 2ME. However, if the cells were plated onto tumour macrophage monolayers, growth occurred in the absence of 2ME (Evans, 1979).

The relationship between macrophage cytotoxic and growth promoting activities is difficult to reconcile. However it may be possible to explain these findings in terms of macrophage heterogeneity. In this connection it is interesting to note that while macrophages from solid murine tumours were cytotoxic in vitro, culture supernatants from these same cells stimulated neoplastic cell proliferation (Evans, 1976). In addition, Mantovani (1978) reported that macrophages freshly isolated from the FS6 fibrosarcoma were cytotoxic in vitro. However, after 24 or 48 hours of culture these cells were found instead to stimulate ^{3}H-thymidine uptake by the target cells. It is possible that distinct subpopulations of macrophages exist within tumours each capable of performing different functions. Under conditions exployed in most in vitro

cytotoxicity assays, highly activated cytotoxic macrophages may prodominate over macrophages which promote tumour growth producing the net effect of cell killing. However, if the effects of cytotoxic macrophages are removed by culture in vitro or by looking at culture supernatants, then the growth promoting activity may be detected.

(c) Suppressor cell activity

For most immune reponses, regulatory mechanisms have been described that limit the generation, magnitude or length of the response. Various different classes of suppressor cell have been implicated, including macrophages (section 3.6). There is some evidence that tumour-associated macrophages may also possess immunosuppressive activity. Cells isolated from regressing MSV-induced tumours have been found to suppress lymphoproliferative responses to mitogens (Holden et al., 1976; Herberman et al., 1980). Similarly, macrophages obtained from human ascitic and solid ovarian tumours have been shown to suppress PHA-induced lymphocyte proliferation (Peri et al., 1982; Haskill et al., 1982). Functional lymphocyte responses may also be suppressed by tumour-associated macrophages.

Gillespie and Russell (1980) studied the effect of tumour-associated macrophages on the in vitro development of cytolytic T lymphocyte activity. Noncytolytic cells obtained

from the lymph nodes draining MSV induced tumours underwent extensive blastogenesis and acquired high levels of antigen-specific cytolytic activity if cultured alone for 4 days (Gillespie and Russell, 1978). However, if macrophages isolated from syngeneic progressively growing SV40-3T3 neoplasms were added to the cultures, the development of cytolytic activity was effectively prevented (Gillespie and Russell, 1980). In contrast, although lymphokine activated peritoneal macrophages also suppressed the development of cytolytic activity at high cell number, at lower cell densities they produced a dramatic and consistent enhancement of the response. Whether the tumour-associated macrophages inhibited the development of cytotoxic lymphocytes or affected the expression of cytotoxicity was not determined.

Some proliferation independent immune responses can also be inhibited by tumour-associated macrophages. The production of MIF by lymphocytes has been shown to be independent of proliferation (Bloom et al., 1972) and mitomycin C treated cells were fully capable of effecting the response (Varesio et al., 1979b). Addition of adherent cells from regressing MSV-induced tumours to cultures of immune spleen cells and RBL-5 tumour cells resulted in abrogation of detectable MIF production (Varesio et al., 1979b). In contrast, normal mouse peritoneal cells had no suppressive activity. Similarly,

macrophages from MSV-induced tumours have been shown to inhibit MIF production by alloimmune spleen cells in response to allogeneic cells (Herberman et al., 1980). Extensive studies have shown that MIF is not absorbed to macrophages, inactivated by material produced by macrophages, nor do macrophages produce a material which counteracts the effects of MIF (Varesio and Holden, 1980a). Varesio and Holden (1980b) demonstrated that suppressor macrophages from regressing MSV-induced tumours inhibited protein synthesis by resting or Con A stimulated lymphocytes. Since protein synthesis is absolutely required for lymphocyte proliferation (Milner, 1978) and lymphokine production (Henney et al., 1974), it is possible that macrophage mediated inhibition of protein synthesis may cause suppression of the development of subsequent immune responses. There are often large numbers of macrophages present within regressing MSV-induced tumours (Holden et al., 1976) and it is possible that the suppressor activity of these cells may account for the inability of lymphocytes from the tumour to be stimulated to produce MIF (Varesio et al., 1979b).

5.3 In vivo significance of tumour-associated macrophages

Considerable care must be taken in extending in vitro findings to the in vivo situation. Because a cell is able to perform a particular function in vitro does not necessarily indicate that it performs the same function in vivo. Isolation

of a specific cell type for study *in vitro* removes that cell from the positive or negative regulatory processes which may occur *in vivo*. Alternatively of course, the isolation procedures used or the conditions employed *in vitro* may not be ideally suited to demonstrate certain cellular functions.

Evans *et al*. (1978) demonstrated the difficulties encountered in relating *in vitro* findings to the *in vivo* situation. They found that after excision of primary fibrosarcomas, subsequent i.p. challenge with tumour cells produced non-specific cytotoxic macrophages and specific cytotoxic T lymphocytes. However, the non-specific killing of tumour cells by peritoneal macrophages *in vitro* did not reflect a similar occurrance *in vivo*. When immune animals were challenged with the immunizing tumour and with graded numbers of non-cross reacting tumour cells, growth of the non-related tumour occurred in all cases and only the specific tumour was rejected. Moreover, the non-specifically cytotoxic peritoneal macrophages did not delay growth of unrelated tumour cells in a Winn assay. Thus no matter how effector cells react *in vitro* or even in the Winn assay, it is still necessary to show that they perform similar functions *in vivo*.

What then is the evidence that tumour-associated macrophages influence tumour development? Probably the best lines of evidence correlate the macrophage content of tumours either

with prognosis or their biological behaviour.

(a) Correlation between mononuclear cell infiltration of human tumours and prognosis

Initial reports demonstrating the presence of mononuclear cells within human tumour masses first appeared during the latter half of the 19th century (reviewed by Underwood, 1974). The gross similarity between the tumour-associated infiltrate and that present within sites of chronic inflammation gave rise to the quite logical theory that cancer originates at sites of previous chronic inflammation. Handley (1907) was the first to suggest a potentially beneficial role for mononuclear cell infiltration of tumours. He studied a single patient with disseminated melanoma and proposed that "the abundant round cell infiltration.....and the absence from this region of a network of small permeated lymphatics are clear indications that reparatory processes, inadequate for cure, are not wanting in melanotic sarcoma."

Many authors have attempted to establish a correlation between mononuclear cell infiltration of tumours and their prognosis. MacCarty (1922) investigated the relationship between various histologically recognizable tumour features and postoperative survival in a group of 293 cases consisting of cancer of the stomach, breast and rectum. He reported a three fold increase in postoperative life provided that lympho-

cyte infiltration, tumour cell differentiation, fibrosis and hyalinization were all present within the primary tumour. Lymphocyte infiltration in the absence of the other features also improved survival prospects although the average length of post operative survival was greater if all the factors were present.

In another retrospective study MacCarty and Mahle(1921) investigated the postoperative life span of 200 gastric cancer patients staged as to degree of spread. The results indicated that for the small number of patients with limited glandular involvement there was a marked survival advantage if tumour cell differentiation and mononuclear cell infiltration were present.

The early studies of MacCarty (1922) and MacCarty and Mahle (1921) were the first to demonstrate an albeit rather weak association between mononuclear cell infiltration and improved prognosis and are particularly important for, unlike most of the more recent investigations, none of the patients received therapy other than surgery. Few recent studies comment on the possible effects of prior radiotherapy or chemotherapy on host cell content or survival. Recently, Underwood (1974) reviewed over 30 publications dealing with non-lymphoid tumours which have appeared since 1921. Most of these reported a positive association between lymphoreticular

infiltration and an improved prognosis, although there were several exceptions.

One of the strongest arguments in favour of an important role of mononuclear cell infiltration in tumour growth comes from studies on medullary carcinoma of the breast. These tumours display highly "malignant" cytological features and in general would be expected to have poor long term survival rates. Moore and Foote (1949) in a study of 1,000 patients who had undergone radical mastectomy identified 52 cases of medullary carcinoma. In most instances such tumours were shown to contain dense lymphoid infiltration. These medullary carcinoma patients exhibited greatly enhanced survival as a group compared to other breast cancers. Only 6 (11.5%) died within 5 years, a figure approximately five times better than was seen with other breast cancers. In addition, although 22 (42.3%) had axillary metastasis at operation, 11 (50%) were well after 5 years compared with a 27% 5 year survival in other types of breast cancer with axillary involvement. Unfortunately, this study was retrospective and used historical controls for survival and some of the figures obtained are at odds with other more extensive studies (Morrison et al., 1973).

Berg (1959) investigated the relationship between inflammation and survival in patients with large anaplastic breast

tumours. He demonstrated that 73% of patients who survived more than 5 years after removal of the primary tumour had shown marked round cell (mainly plasma cell) infiltration at the junction of tumour and normal breast while only 30% of patients who survived less than three years had exhibited a similar infiltration. Berg concluded that the association between good prognosis and inflammation was probably the result of the lower metastatic potential of large anaplastic tumours associated with plasma cell infiltrate.

A large number of studies have demonstrated a strong correlation between the content of host immune cells and prognosis in Hodgkin's lymphoma. For example, Keller et al. (1968) subgrouped 176 previously untreated cases of Stage I and II Hodgkin's disease as to whether they exhibited lymphocyte predominance, nodular sclerosis, mixed cellularity or lymphocyte depletion. Lymphocyte predominance was observed in 5% of cases and such patients exhibited greatly improved prognosis as a group, with approximately 90% surviving longer than 5 years. In contrast, only 40% of patients with lymphocyte depletion survived 5 years. Similar results have been obtained by many other groups (reviewed by Underwood, 1974).

It appears that at least for certain human tumours there is a direct correlation between the degree of host cell infiltration and an improved clinical prognosis. However, many

exceptions have been reported and some authors disappointed by inconsistant results have rejected the concept entirely (Champion et al., 1972; McGovern et al., 1973; Morrison et al., 1973). However, it would indeed be surprising if it were always possible to correlate a complex biological phenomemon such as survival with any single characteristic in all tumours. In addition, in many studies the nature of the infiltrate was not defined or loose generalized descriptions such as round cell or lymphoreticular were used. These descriptions do not take into account the functional heterogeneity that exists within populations of lymphocytes or macrophages nor do they consider the variable effects different tumours may have on immune cell function.

Virtually all reports correlating survival with mononuclear cell infiltration have been concerned with the characteristics of a surgically removed primary tumour. However, it is metastatic disease which kills most patients (Clark, 1979). This raises the question of how tumour-associated macrophages bring about improved prognosis. One possible explanation is that these cells benefit the host by preventing or reducing metastasis.

Lauder et al. (1977) suggested that the close association of macrophages and tumour cells observed in primary breast carcinomas may have retarded tumour cell emigration

and contributed to the lack of metastasis in those patients with prominent host cell infiltrates. Similarly, Pelouze and Bonenfant (1979) showed that gastric carcinomas infiltrated by high numbers of host cells produced fewer nodal metastases and an increased duration of survival as compared to similar tumours infiltrated by low numbers of host cells (reviewed by Eccles, 1982). The possible relationship between host cell content and metastasis has received considerable attention in animal studies.

(b) Correlation between macrophage infiltration of animal tumours and metastasis

Evidence that mononuclear cell infiltration of tumours influences their capacity for spontaneous metastasis can be no more than circumstantial. It is however a frequent finding that tumours with the lowest incidences of spontaneous metastases tend to be immunogenic and contain the highest numbers of host cells, particularly macrophages. Such a correlation has been demonstrated for rat sarcomas (Eccles and Alexander, 1974), mouse sarcomas (Mantovani, 1978), mouse lymphomas (Davey et al., 1979) and rat gliomas and sarcomas (Morantz et al., 1979; Moore and Moore, 1977a). In studies where tumour immunogenicity but not host cell content was reported, various investigators have shown that highly immunogenic rat mammary carcinomas (Kim, 1970) and mouse colorectal carcinomas (Belnap et al., 1979) and sarcomas (Fidler et al., 1979) yielded fewer spontaneous

metastases than did similar poorly immunogenic tumours.

There are of course many exceptions to this rule. For instance, Eccles (1982) found groups of Bittner factor positive mouse mammary adenocarcinomas and chemically induced squamous cell carcinomas in which negligibly or weakly immunogenic tumours containing low to moderate numbers of macrophages failed to develop spontaneous metastases. It must be remembered however that metastasis is a complex biological phenomenon which depends on both the characteristics of the tumour cells and the host immune response. Presumably in some circumstances tumour characteristics alone may prevent or limit the possibility of spontaneous metastases developing (Sugarbaker, 1979). Thus while low levels of infiltrating cells are not necessarily associated with tumours of high metastatic potential there are few examples in animal studies, of tumours containing a dense host cell infiltrate successfully producing high incidences of overt metastasis. Although these observations do not establish causality they are strongly suggestive of a role for tumour-associated host cells, particularly macrophages, in preventing metastasis.

The major advantage of animal studies is the ability to manipulate various immunological parameters. Thus the importance of tumour-associated macrophages to the development of spontaneous metastases can be investigated by

increasing or decreasing the number of such cells. In some tumour systems T-lymphocyte depletion produces a considerable decrease in the number of tumour-associated macrophages and an associated increase in the incidence and/or distribution of metastases. This effect has been documented for rat sarcomas grown in animals T lymphocyte depleted by thymectomy plus whole body irradiation (Eccles and Alexander, 1974) or Cyclosporin A treatment (Eccles et al., 1980) and in congenically athymic animals (Eccles, 1980; Eccles et al., 1979). Similar results were also obtained for mouse sarcomas or lymphomas grown in congenitally athymic animals (Eccles, 1982) or Cyclosporin A treated animals (Eccles et al., 1980) and mouse mammary adenocarcinomas grown in animals T-lymphocyte depleted by thymectomy and irradiation (Parthenais and Haskill, 1979). Reconstitution of T-lymphocyte depleted animals with lymphoid cells usually restored metastasis to the levels found in control animals (Eccles, 1978; Pimm and Baldwin, 1978; Parthenais and Haskill, 1979).

Thus it appears that manipulation of T cell responses reduces tumour-associated macrophage number and may affect metastasis, suggesting that these variables are somehow related. It should however be noted that the processes used to alter the macrophage content of a tumour frequently alter other factors that might influence metastasis, in particular lymphocyte-mediated responses.

Assuming for a moment that tumour-associated macrophages are important in controlling metastasis how might they produce this effect? Certainly, if it were possible to prevent malignant cells from leaving the primary tumour site, metastasis would not occur. It is tempting to consider that high levels of tumour-associated macrophages, while failing to prevent local growth may still prevent or inhibit tumour cell emigration. If this mechanism does occur then one might expect macrophages from highly immunogenic, non-metastatic tumours to be more cytotoxic than those from weakly immunogenic, metastatic tumours. Mantovani (1978) found that the highly immunogenic non-metastatic murine FS6 sarcoma contained more than twice the number of macrophages than its weakly immunogenic, spontaneously metastatic subline mFS6. In addition, while macrophages from the FS6 tumour inhibited the growth and DNA synthesis of tumour cells in vitro, macrophages obtained from the mFS6 tumour non-specifically enhanced the proliferative activity of tumour target cells. However, opposite results have been found in other tumour systems. For example, macrophages isolated from the highly immunogenic, non-metastatic rat HSBPA fibrosarcoma were poorly cytotoxic in vitro (Eccles and Alexander, 1974). In contrast macrophages from the poorly immunogenic, metastatic rat HSN and ASBP1 fibrosarcomas exhibited strong non-specific cytotoxic activity (Evans, 1975). Most recently, Loveless and Heppner (1983) compared the macrophage content and function of a series of transplanted mouse

mammary tumours originally derived from a single spontaneously arising tumour. No correlation was found between macrophage content and metastatic capacity. Moreover, macrophages from every metastatic tumour used were found to be cytotoxic whereas only 6 out of 17 non-metastatic tumours contained cytotoxic macrophages.

(c) Correlation between macrophage content of tumours and regression

Spontaneous regression of established primary neoplasms in humans is a relatively rare event. There has however been infrequent observations of spontaneous regression of malignant melanoma in which partial or total disappearance of primary tumours has been clearly documented. Tumour regression was frequently accompanied by a dense host cell infiltrate (Burg and Braun-Falco, 1972; McGovern et al., 1973) although several reports failed to find such an association (Jones et al., 1968; Cochran, 1969). Tumour cell imprint preparations from a regressing melanotic lesion revealed numerous large macrophages in juxtaposition to melanoma cells (The et al., 1972). Epstein et al. (1973) reported a series of melanoma patients with skin metastases in whom removal of a primary melanoma was associated with the appearance of multiple prominent halo nevi. However, on this occasion lymphocytes and not macrophages were observed around typical degenerating melanoma cells.

In two well established murine tumour systems, the mammary adenocarcinoma T1699 (Haskill et al., 1975b) and the Moloney virus-induced sarcoma (Russell and Cochrane, 1974; Holden et al., 1976), spontaneous regression was associated with the accumulation of lymphocytes, macrophages and granulocytes. Both tumour-associated lymphocytes and macrophages exhibited direct cytotoxic activity in vitro. Moreover, macrophages from regressing T1699 tumours were also capable of killing tumour targets by ADCC (Haskill and Fett, 1976).

For some regressing tumours, histological studies show a close association between infiltrating macrophages and tumour cells. For example, in a regressing transplanted rat tumour, numerous macrophages were observed with long processes closely apposed to tumour cells, with all stages of phagocytosis of apparently intact tumour cells evident (Carr et al., 1974). Analogous appearances have been noted in regressing leukaemic intradermal tumours and hepatomas (Snodgrass and Hanna, 1973; Feldman et al., 1974).

If tumour-associated macrophages do have the potential to destroy neoplastic cells, then it is logical to assume that experimental procedures which increase either their number, or functional activity will have an inhibitory effect on tumour growth. Indeed, treatment of tumour masses by intra-

lesional injection of BCG or C.parvum is based on the assumption that these procedures will increase both the number and activity of tumour-associated macrophages. Moreover, such treatments have on occasion been shown to produce dramatic regression of animal (Zbar et al., 1972; Likhite, 1974) and human (Morton et al., 1970, 1974; Holmes et al., 1976) tumours. In addition, several studies have demonstrated that bacterial adjuvants injected intratumourally or admixed with a tumour cell inoculum greatly increased the macrophage content of certain rat or mouse sarcomas and produced a decrease in the number or incidence of spontaneous metastases (reviewed by Eccles, 1982).

Moore and McBride (1980) reported that regression of a murine methylcholanthrene-induced fibrosarcoma following a single intraveneous (i.v.) injection of C.parvum was accompanied by the appearance within the tumour of a population of small highly activated macrophages.

Liposomes constitute a convenient carrier to target biologically active materials to macrophages. Following i.v. administration, the vast majority (80-90%) of liposomes are taken up by macrophages (Poste et al.,1982). Moreover, liposome-encapsulated mediators of activation such as lymphokines or the synthetic agent N-acetyl-L-alanyl-D-isoglutamine (MDP) are able to induce cytolytic activity in macrophages

in vivo and *in vitro* (reviewed by Fidler and Poste, 1982). It has been reported that multiple i.v. injections of liposomes containing lymphokines (Fidler, 1980; Fidler *et al.*, 1980; Hart *et al.*, 1981; Fidler *et al.*, 1982; Poste *et al.*, 1982) or MDP (Fidler *et al.*, 1980; Fidler, 1981; Fidler *et al.*, 1981; Fidler *et al.*, 1982) resulted in significant destruction of established murine melanoma lung metastases. It was assumed that the mechanism of this effect involves the localization of activated macrophages within the metastatic lesions (Fidler and Poste, 1982).

In a recent study, Obrist and Sandberg (1983) reported that the number of macrophages infiltrating peritoneal hepatomas was significantly elevated by the i.p. injection of a covalent conjugate of the chemotactic peptide, formylmethionylleucyl-phenylalanine and a monoclonal antibody reactive with cell surface antigens on the hepatoma cells. This is a particularly important study as it provides a mechanism whereby macrophages can be specifically attracted to a tumour mass. The use of such chemotactic conjugates in conjunction with mediators of macrophage activation could perhaps constitute an effective method of immunotherapy.

Whether accumulation of host cells within a tumour actually causes regression or is only circumstantial is often not clear. Treatment of sarcoma bearing mice with daily

azathioprine injections resulted in an increase in the proportion of tumour-associated T lymphocytes and macrophages. However, individually neither macrophages or lymphocytes were cytotoxic *in vitro* (Evans, 1976). Thus it remains questionable whether tumour regression was the result of cell-mediated cytotoxicity or of drug action alone. Similarly, it was recently reported that during cyclophosphamide-induced tumour regression, there was an accumulation and infiltration of macrophages throughout the tumour mass (Evans, 1980; Evans *et al.*, 1980). However, it was apparent in these studies that although the tumour mass became smaller, there was an actual increase in the number of tumourogenic cells within the regressing mass and most tumours eventually showed renewed growth (Evans, 1982).

In summary, it seems that although tumour regression is commonly accompanied by a dense host cell infiltrate particularly macrophages, there is insufficient evidence at present to suggest that such host cells always cause the regression. Indeed what little evidence there is available suggests instead that these cells are often involved in other reactions such as clearance of debris (Key, 1983).

CHAPTER II - AIMS

From a historical point of view, the development of techniques for the disaggregation of solid tumour tissue (Evans, 1972) and the separation of highly enriched populations of tumour-associated macrophages (Evans, 1973), came at a time when interest was focussed on the ability of macrophages from other sources (mainly the peritoneal cavity) to destroy neoplastic cells in vitro (reviewed by Evans and Alexander, 1976). Consequently, most studies on the functional capabilities of tumour-associated macrophages have tended to look only at the cytotoxic activity of these cells (reviewed by Evans and Haskill, 1983). There is however increasing evidence that macrophages play a pivitol role in many diverse immunobiological reactions (reviewed by Dougherty and McBride, 1984) and their presence within the tumour mass suggests various ways in which they may interact with the neoplasm.

In recent years a vast amount of data has been collected implicating the macrophage as an essential accessory cell in the induction, expression and regulation of humoral and cell-mediated immune responses (reviewed by Unanue, 1981; Unanue et al., 1984). However, at present, very little is known about the accessory cell activity of tumour-associated macrophages. It was the aim of the present study to identify and characterize the macrophages infiltrating the highly immunogenic methylcholanthrene-induced murine fibrosarcoma FSA-R and

investigate the accessory cell activity of these cells in well defined *in vitro* assay systems.

CHAPTER III - MATERIALS AND METHODS

1.0 BUFFERS, MEDIA AND STAINS

Unless otherwise stated all chemicals used in preparation of buffers, media and stains were analytical grade obtained from BDH Chemicals Ltd., Poole, England.

1.1 Cacodylate buffer, pH 6.9, 0.28M

60g of sodium cacodylate (Fisons Scientific Apparatus, Loughborough, England) was dissolved in 950 ml distilled water and the pH adjusted to 6.9 with approximately 11 ml 1N HCC. The final volume of the solution was made up to 1 litre with distilled water.

1.2 Dulbecco's phosphate buffered saline (modified) (D.PBS)

D.PBS was prepared by dissolving 8.0g NaCl, 0.2g KCl, 1.08g $Na_2 HPO_4$, 0.2g KH_2PO_4, 0.89g $CaCl_2.2H_2O$ and 0.203g $MgCl_2.6H_2O$ up to 1 litre with distilled water.

1.3 Hank's balanced salt solution (HBSS)

HBSS was made up from a powdered concentrate purchased from Oxoid Ltd., Basingstoke, England.

1.4 Tris buffered saline, pH 7.6 (TBS)

A stock solution of TBS was prepared by dissolving 6.05g

of 2-amino-2-(hydroxymethyl) propane - 1,3-diol (Tris buffer) (Fisons Scientific Apparatus, Loughborough, England) and 8.5g of NaCl in 950 ml distilled water and the pH adjusted to 7.6 with approximately 40 ml of IN HCl. The final volume of the solution was made up to 1 litre with distilled water. The stock solution was stored for up to 1 month at $4^{o}C$ and diluted 1 in 10 with normal saline as required.

1.5 Tissue culture medium

RPMI 1640 medium containing 2.0g/l Na HCO_3 (Flow Laboratories Ltd., Irvine, Scotland) was supplemented with 10% heat inactivated foetal calf serum (FCS) (Gibco Bio-Cult Ltd., Paisley, Scotland), 2mM glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 200 units/ml penicillin (Sigma Chemical Company Ltd., Poole, England) and 100µg/ml streptomycin (Sigma Chemical Company Ltd., Poole, England).

1.6 Giemsa stain

Giemsa stain was diluted 1 in 10 with D.PBS. Cells were stained for 2 minutes followed by 5 minutes destaining in D.PBS.

1.7 Mayer's haematoxylin stain

3.0g haematoxylin, 0.4g $NaIO_3$ and 100g $AlK(SO_4)_2 . 12H_2O$ were made up to 2 litres with distilled water. To this solution was added 100g $CCl_3.CH(OH)_2$ and 2g of citric acid. Immediately

before use, the haematoxylin stain was "ripened" by boiling for 5 minutes. Cells were stained for 1 minute at room temperature.

1.8 Trypan Blue

A 0.05% (w/v) solution of trypan blue was prepared in saline. To perform a viable cell count 1 part cell suspension was added to 9 parts stain.

1.9 White cell diluting fluid

A stock solution was prepared by adding 0.1g gentian violet to 100 ml 1% acetic acid. To perform a total nucleated cell count, the stock solution was diluted 1 in 10 with 1% acetic acid and 1 part cell suspension was added to 9 parts stain.

2.0 REAGENTS

2.1 Antisera

Hybridomas secreting the M1/70.15.11.5 rat monoclonal antibody reactive with the macrophage-granulocyte "specific" antigen Mac-1 (Springer et al., 1979) or the 10-2.16 mouse monoclonal antibody reactive with I-A^k (Oi et al., 1978) were obtained from the American Type Culture Collection, Rockville, Maryland, USA. Rat anti-mouse Thy-1.2 was a kind gift from Dr H. S. Micklem, Department of Zoology, University of Edinburgh. ATH anti-ATL, a broadly-reactive anti-Ia^k antiserum was purchased from Sera-Lab Ltd., Crawley Down, England. Hyperimmune rabbit anti-CRBC antiserum was a kind gift from Dr W. H. McBride, Department of Bacteriology, University of Edinburgh.

2.2 Complement

Pooled normal guinea pig serum served as a source of complement for use in haemolytic plaque assays and anti-Ia cytotoxicity. In order to remove any naturally occurring antibodies, the serum was absorbed for 1 hour at 4^oC with 30-40% (v/v). packed erythrocytes (for plaque-forming cell assay) or an equivalent volume of packed enzymatically disaggregated FSA-R tumour cells (for anti-Ia cytotoxicity). The cells were then spun down, and the serum aliquoted and stored in liquid nitrogen.

2.3 Erythrocytes

Calf red blood cells (CRBC) in Alsever's solution were purchased from Tissue Culture Services, Slough, England. Sheep red blood cells (SRBC) in Alsever's solution were a kind gift from Mr C. Birrells, Moredun Research Institute, Edinburgh, Scotland.

3.0 MICE

Male C3Hf/Bu Kam mice aged 8-18 weeks were bred and maintained in the animal unit, Department of Bacteriology, University of Edinburgh. In some experiments syngeneic athymic nude (nu/nu) mice and their phenotypically normal heterozygous (nu/+) littermate controls were used. These mice were derived from breeding stock kindly provided by Mr C. McLean, Department Radiation Oncology, U.C.L.A., Los Angeles, USA.

4.0 TUMOUR

The FSA-R tumour used in the present study was a well characterized, highly immunogenic, methylcholanthrene-induced fibrosarcoma syngeneic for C3H f/Bu Kam mice (Suit and Kastelan, 1970; Milas *et al.*, 1974; Peters *et al.*, 1978; McBride *et al.*, 1980; Moore and McBride, 1980; Howie and McBride, 1982b). It was maintained by serial passage *in vivo* and used at transplant generation 8-18. Experimental tumours were induced by s.c. inoculation of 5×10^5 viable enzymatically disaggregated tumour cells into the right flank.

5.0 IDENTIFICATION, CHARACTERIZATION AND SEPARATION OF MACROPHAGES

5.1 Preparation of antibody-coated erythrocytes

One ml of 5% (v/v) washed CRBC in HBSS was mixed with an equal volume of hyperimmune rabbit anti-CRBC antiserum diluted to one-half maximum subagglutinating concentration in HBSS, and the mixture incubated at 37°C for 30 minutes. The antibody-coated erythrocytes (EA) were washed three times and finally resuspended at 2% (v/v) in HBSS.

5.2 Detection of Fc receptor-positive cells in sections of tumour tissue

Frozen sections were cut from tumour tissue using a Teddington Type QR cryostat (Bright Instrument Company Ltd., Huntingdon, England). Tumours were excised and rapidly frozen in liquid nitrogen. A suitably sized block of tissue was cut from the frozen tumour, mounted on the cryostat specimen plate in Cryo-M-Bed embedding medium (Bright Instrument Company Ltd., Huntingdon, England) and rapidly cooled using Arctic spray (Warecare Ltd., Leic., England). The specimen plate was mounted in the cryostat and a large number of 6 μm thick serial sections cut. These cryostat sections were mounted on clean uncoated glass slides and rapidly dried at 37°C for 45 minutes.

To detect Fc receptor-positive cells, tissue sections were

overlayed with 0.5% (v/v) EA in HBSS and incubated at room temperature for 45 minutes. After incubation, non-adherent cells were removed by immersion with gentle agitation in HBSS. The sections were then fixed in 2% glutaraldehyde (BDH Chemicals Ltd., Poole, England) in cacodylate buffer and the distribution and extent of EA absorption determined.

5.3 Preparation of enzymatically disaggregated tumour cell suspensions

Enzymatically disaggregated tumour cell suspensions were prepared as previously described by Moore and McBride (1980). Briefly, tumours were excised and any obviously necrotic areas removed. Non-necrotic tissue from three tumours was pooled and thoroughly minced using a No 11 scalpel blade (Swan Morton Ltd., Sheffield, England). The minced material was washed three times with HBSS to remove cell debris and erythrocytes. Approximately 1g of washed tumour tissue was added to 20 ml of 0.05% Dispase (Boehringer, Mannheim, W. Germany) and 0.002% Deoxyribonuclease-1 E.C. 3.1.4.5 (DNase) (BDH Chemicals Ltd., Poole, England) in HBSS and stirred briskly for 45 minutes at room temperature. Any non-disaggregated material was allowed to settle for 3-5 minutes. The released tumour cells present in the supernatant were drawn off by pipette, centrifuged at 200g for 10 minutes and washed twice with HBSS.

5.4 Preparation of mechanically disaggregated tumour cell suspensions

Approximately 1g of minced tumour tissue (prepared as in section 5.3) was added to 5g of 3mm diameter glass beads and 20ml HBSS. This mixture was shaken vigorously for 45 minutes at room temperature. The glass beads and remaining tumour tissue fragments were allowed to settle for 3-5 minutes. Released tumour cells present in the supernatant were drawn off by pipette, centrifuged at 200g for 10 minutes and washed twice with HBSS.

5.5 Collection of resident or C.parvum-elicited peritoneal cells

Normal mice, or mice which had received an intraperitoneal injection of 0.25mg C.parvum (Lot 776/1, Wellcome Research Laboratories, Beckenham, England) in 0.25ml saline 4 days previously, were killed by cervical dislocation and their peritoneal cavities vigorously lavaged with 10ml HBSS. The cell suspensions obtained were centrifuged at 200g for 10 minutes.

5.6 Separation of macrophages

Macrophages were enriched from enzymatically disaggregated tumour cell suspensions or populations of resident or C.parvum-elicited peritoneal cells by virtue of their capacity to adhere tightly to solid substrates.

Enzymatically disaggregated tumour cells were resuspended

in HBSS containing 0.1% Dispase and 20% FCS, a mixture which effectively prevents lymphocytes and neoplastic cells from adhering. One ml aliquots of the cell suspension were placed in the wells of a Nunc 24 well tissue culture plate (Gibco Bio-Cult Ltd., Paisley, Scotland) and the plate incubated at 37°C for 30 minutes. Following the incubation period non-adherent cells were removed by vigorously washing the cell monolayers three times with HBSS.

Macrophages were enriched from populations of resident or C.parvum-elicited peritoneal cells by the same procedure except that these cells were resuspended in HBSS containing 20% FCS but no Dispase.

Macrophage monolayers for use in the immunoperoxidase assay were prepared using the same technique except that these cells were adhered to 13mm diameter glass coverslips placed in the bottom of tissue culture wells.

Adherent cells were quantitated by inverted phase microscopy. The area of a field was determined for each microscope objective, using a calibrated graticule. The average number of cells present per field was then determined for 10 randomly selected, well dispersed fields. A suitable magnification was chosen such that each field contained 25-75 cells. Since the area of each field was known, it was possible to calculate the number of cells

present in the culture simply by multiplying the average number of cells per field by the number of fields which make up the area of the culture vessel.

5.7 Detection of Fc receptor-positive cells in cell suspensions

Enzymatically disaggregated tumour cells and resident or C.parvum-elicited peritoneal cells were resuspended in HBSS at a concentration of 2×10^6 viable cells/ml. 0.5ml of each cell suspension was placed in a 3ml plastic test tube, 0.5ml of EA suspension was added and the mixture centrifuged at 100g for 5 minutes. Pelleted cells were incubated at room temperature for 30 minutes without disturbing. After the incubation period, the pellet was gently disrupted by shaking and the cells loaded into an Improved Neubauer Haemocytometer. A total of 200 cells was counted to determine the percentage of EA rosette-forming cells binding three or more erythrocytes.

5.8 Detection of Fc receptor-positive cells in monolayers of adherent cells

Monolayers of adherent cells were overlayed with 0.5ml HBSS and 0.25ml EA suspension was added. Plates were centrifuged at 100g for 5 minutes and incubated at room temperature for 30 minutes. Non-adherent EA were removed by washing monolayers three times with HBSS and a total of 200 cells was counted to determine the percentage of EA rosette-forming cells binding three or more erythrocytes.

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5.8 Detection of Fc receptor-positive cells in monolayers of adherent cells

Monolayers of adherent cells were overlayed with 0.5ml HBSS and 0.25ml EA suspension was added. Plates were centrifuged at 100g for 5 minutes and incubated at room temperature for 30 minutes. Non-adherent EA were removed by washing monolayers three times with HBSS and a total of 200 cells was counted to determine the percentage of EA rosette-forming cells binding three or more erythrocytes.

5.9 Identification of phagocytic cells by latex particle uptake

Enzymatically disaggregated tumour cells and resident or C.parvum-elicited peritoneal cells were resuspended at 1 x 10^6 viable cells/ml in tissue culture medium. Latex particles, 0.81 μm in diameter (Difco Laboratories, Detroit, Michigan, USA) were washed three times in HBSS and resuspended at 5 x 10^8 particles/ml in tissue culture medium. One ml aliquots of each cell suspension were placed in Nunc "minisorp" polyethylene tubes (Gibco Bio-Cult Ltd., Paisley, Scotland), 100ul of the latex particle suspension was added and mixture incubated for 1 hour at 37°C in an atmosphere containing 5% CO_2. The cells were washed three times with HBSS, resuspended in 1 ml of fresh tissue culture medium and incubated at 37°C for a further 30 minutes to allow ingestion of attached particles. A total of 200 cells was then counted under phase to determine the percentage of cells which had taken up 5 or more latex particles.

Phagocytic cells were also identified in monolayers of adherent cells using the same procedure as described above.

5.10 Preparation of cytocentrifuged cell smears

Cell smears were prepared using a Cytospin cytocentrifuge (Shandon Southern Instruments, Runcorn, England). Cells were resuspended in HBSS containing 10% FCS at 2 x 10^5 cells/ml. 0.2ml aliquots of the cell suspension were added to the cyto-

centrifuge chambers and spun at 1500 rpm for 5 minutes.

5.11 Immunoperoxidase technique

An indirect immunoperoxidase technique was used to determine antigen expression on cytocentrifuged cell smears or monolayers of cells adherent to glass coverslips. All procedures were carried out at room temperature in a humidified atmosphere.

Cells were fixed in 1% paraformaldehyde (Fisons Scientific Apparatus, Loughborough, England) in cacodylate buffer for 20 minutes. The fixative was removed and the cells washed three times in TBS. Endogenous peroxidase activity was blocked by incubating the fixed cells in methanol containing 0.3% hydrogen peroxide (BDH Chemicals Ltd., Poole, England) for 15 minutes. The cells were then washed three times in TBS and any available Fc receptors blocked by incubating with TBS containing 20% normal rabbit serum (NRS) (Scottish Antibody Production Unit, Carluke, Scotland) for 15 minutes. After this incubation period, the fluid was gently drawn off and the cells incubated with the appropriate hybridoma culture supernatant for 30 minutes. The cells were then washed for three 5 minute periods to remove unbound antibody, and incubated for 10 minutes with the appropriate peroxidase-conjugated rabbit second antibody (Dakopatts, Copenhagen, Denmark) diluted 1/80 in TBS containing 2% NRS. The cells were then washed for three 10 minute periods and cell-bound peroxidase-conjugated antibodies visualised using

the substrate 3, 4, 3', 4'-tetra-aminobiphenyl hydrochloride (DAB) (BDH Chemicals Ltd., Poole, England). The substrate was made up by dissolving 4mg DAB in 20 ml stock TBS containing 10µl 30% (v/v) hydrogen peroxide. Cells were incubated with the substrate for 4 minutes, thoroughly washed with distilled water, counterstained with haematoxylin and mounted in D.P.X.

6.0 DETERMINATION OF MACROPHAGE ACCESSORY CELL ACTIVITY

6.1 CRBC priming

Animals were injected i.p. with 0.1ml 1% (v/v) CRBC in HBSS on two occasions 4 weeks apart. Spleens were used as a source of CRBC-specific T_H cells 2-4 weeks after the last injection.

6.2 Separation of splenic T lymphocytes

T lymphocytes were purified by passage of spleen cells over a nylon wool column as described by Julius et al. (1973). Briefly, 1g of scrubbed nylon fiber (Fenwal Laboratories, Deerfield, Illinois, USA) was packed into a plastic 5ml disposable syringe (Becton Dickinson and Co. Ltd., Ireland), giving a column approximately 3 cm high. Columns were rinsed with 25ml tissue culture medium and incubated at 37°C for 1 hour in a 5% CO_2 atmostphere. After the incubation period the columns were allowed to run dry and 1 x 10^8 spleen cells in 1ml of tissue culture medium were gently layered onto the top of the column and allowed to penetrate the nylon wool. The columns were then sealed using the syringe plunger and incubated at 37°C for 1 hour in a 5% CO_2 atmostphere. Non-adherent cells were eluted by washing columns with tissue culture medium pre-warmed to 37°C. Only the first 15 ml eluted from the column were collected. Approximately 10-15% of the cells initially applied were recovered following this procedure and 85-95% of these were found to be Thy-1.2 positive by immunofluorescence (Howie and McBride, 1982b).

6.3 Depletion of accessory cells from spleen cell suspensions

Spleen cells were depleted of accessory cells by passage through a column of Sephadex G-10. The method used was a slight modification of that described by Ly and Mishell (1974).

250g of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) was added to 2 litres of saline and allowed to swell overnight at 4°C. After this period, the saline was removed from the settled Sephadex by suction and the gel resuspended in a volume of fresh saline that was approximately four times the estimated bed volume. The Sephadex was allowed to settle for 15 minutes and any unsettled fine particles were removed by suction. This process was repeated three or four times or until the majority of fine Sephadex particles had been removed. A volume of saline equal to 30-50% of the estimated bed volume was added to the Sephadex. The slurry produced was aliquoted and autoclaved at 110°C for 40 minutes with slow exhaust. The sterile Sephadex was stored at room temperature until required.

Approximately 1g of glass wool (May and Baker Ltd., Dagenham, England) was tightly packed into the bottom of a 60ml disposable plastic syringe (Becton Dickinson and Co. Ltd., Ireland) and sufficient Sephadex G-10 added to give a packed column up to the 50ml mark on the syringe. The column was washed with 100-150ml of HBSS containing 5% FCS. After the last of the HBSS plus 5% FCS had been added, the top of the

Sephadex was stirred with a pipette to give a level top to the column. When all the fluid had penetrated the column, 5 ml of spleen cell suspension containing 1 x 10^8 viable cells/ml was carefully added. Once the cells had penetrated the column, small amounts of HBSS plus 5% FCS were added until the cell band was midway down the column. At this point the syringe was filled to the top with HBSS plus 5% FCS. When the cell band reached the glass wool layer the next 15 ml of column effluent was collected. The cell suspension was allowed to stand for 2-3 minutes to allow any contaminating Sephadex to settle before the cells were transferred to another container and centrifuged at 200g for 10 minutes.

6.4 Depletion of Ia-positive cells from adherent cell monolayers

Monolayers of adherent cells were overlayed with 0.5ml of ATH anti-ATL antiserum diluted 1/20 in HBSS and incubated for 30 minutes on ice. The antiserum was drawn off, replaced by absorbed guinea pig serum diluted 1/20 in HBSS and the plates incubated at 37°C for 1 hour. At the end of this time, monolayers were washed three times with HBSS and the number of adherent cells remaining determined by inverted phase microscopy.

6.5 Separation of enzymatically disaggregated tumour cells by centrifugal elutriation

Enzymatically disaggregated tumour cells were separated into populations of differing modal diameter using the Beckman

JE-6 elutriator rotor fitted with a standard horizontal chamber and driven by a modified J2-21 centrifuge. Fluid was pumped through the system using a Masterflex peristaltic pump with fine adjustable velocity control. Cells were elutriated in D.PBS at room temperature. The rotor was maintained at a constant speed of 2000 rpm and 1 x 10^8 viable cells were loaded into the chamber. The pump speed was then progressively increased and 5 150ml fractions were collected at the flow rates indicated in the results section. The cells present in each fraction were spun down at 200g for 10 minutes, resuspended in 10ml tissue culture medium and their size distribution determined using a Coulter Channelyser system (Coulter Electronics Ltd., Luton, England) which was previously calibrated using latex beads of known size. The cellular composition of each fraction was determined from Giemsa stained cytocentrifuge preparations.

6.6 Preparation of macrophage conditioned medium

Monolayers of tumour-derived adherent cells were prepared as described in Section 5.6. Monolayers were overlayed with 1 ml of tissue culture medium and incubated at 37°C for 24 or 48 hours in a 5% CO_2 atmostphere. Supernatants from at least 6 replicate wells were pooled, centrifuged at 450g for 20 minutes to remove any cell debris and stored in liquid nitrogen until required.

6.7 Trinitrophenylation of tumour cells and erythrocytes

Irradiated (5000 rads) enzymatically disaggregated FSA-R tumour cells at 10^6 cells/ml or washed CRBC or SRBC at 10% (v/v) were mixed with an equal volume of cacodylate buffer containing 2mg/ml 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Eastman Kodak Co., Rochester, NY, USA) and incubated for 10-15 minutes at room temperature. At the end of this time, the reaction was halted by the addition of 10mg/ml glycylglycine (Sigma Chemical Company, Poole, England). The trinitrophenyl (TNP)-coupled cells were spun down and washed 3 times with HBSS. TNP-coupled tumour cells were resuspended in HBSS containing 10% dimethyl sulfoxide and 20% FCS, aliquoted and stored in liquid nitrogen. TNP-coupled erythrocytes were stored at $4^{\circ}C$ in HBSS and used within 3 days.

6.8 Determination of macrophage accessory cell activity

Macrophage accessory cell activity was examined in the generation of PFC responses against CRBC or TNP, in the presence or absence of added T_H cells. Triplicate 2ml cultures in RPMI 1640 tissue culture medium were set up as described in the results section incubated at $37^{\circ}C$ in a 5% CO_2 atmosphere and the PFC responses generated were normally determined on day 4.

6.9 Detection of haemolytic PFC

The method used was essentially that previously described by Cunningham (Cunningham, 1965; Cunningham and Szenberg, 1968).

Indicator erythrocytes, lymphoid cells and complement were mixed together, placed in a microchamber and allowed to settle as a monolayer. Antibodies released by single lymphocytes bind to surrounding erythrocytes which are subsequently lysed as the result of complement activation thereby producing plaques. The method is relatively simple, economic and sensitive and permits the enumeration of antibody-producing cells even when they constitute a small minority of the total lymphocyte population.

Microchambers were constructed from pre-cleaned glass microscope slides. Approximately 25 slides were aligned along the edge of a bench and strips of 5mm wide, double-sided, pressure sensitive Scotch tape were placed along the middle and both ends of the row of slides. Other pre-clean slides were placed directly on top of the taped slides thereby forming a microchamber between the two slides. Microchambers prepared in this fashion had a capacity of approximately 175µl.

To determine the number of PFC generated in vitro, the cells present in each tissue culture well were gently resuspended and the cell suspensions transferred to small plastic centrifuge tubes. The cells were centrifuged at 250g for 5 minutes and resuspended in 1ml of HBSS containing 5% of FCS. 50- 150µl of the cell suspensions were added to 20µl 20% (v/v) indicator erythrocytes and 10µl absorbed guinea pig serum and the mixture made up to a final volume of 200µl with HBSS plus 5% FCS. With an appropriate

pipette a measured volume of this mixture was loaded into a microchamber. The edges of the chamber were sealed with molten wax and the slides incubated at 37^{o}C for 1 hour. The haemolytic plaques produced were quite visible with the naked eye but for accuracy were counted using a low powered microscope with oblique illumination. CRBC indicator erythrocytes were used to determine anti-CRBC PFC responses while anti-TNP responses were determined by subtracting the number of background PFC found using SRBC indicator cells from the number of PFC found using TNP-SRBC indicator cells.

7.0 STATISTICS

The statistical significance of data was determined using Student's t-test.

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The statistical significance of data was determined using Student's t-test.

CHAPTER IV - RESULTS

SECTION I - IDENTIFICATION, CHARACTERIZATION AND SEPARATION OF TUMOUR-ASSOCIATED MACROPHAGES

1.0 TUMOUR CHARACTERIZATION

The FSA-R tumour used in these studies is a highly immunogeneic methylcholanthrene-induced fibrosarcoma. It was maintained in syngeneic C3H f/Bu Kam mice by serial s.c. passage of $5x10^5$ viable tumour cells. With this inoculum 100% of animals developed tumours. Palpable tumours first became evident around 7 days post inoculation and grew progressively killing all animals by day 32 (Figure 1). Tumour masses were discrete and circumscribed with minimal invasion of surrounding tissues. There was no evidence of obvious metastatic disease, although tumour cells could occasionally be cultured from the spleen of tumour-bearing animals. Tumours less than 20 mm in diameter were usually firm with only a few small necrotic areas. After exceeding 20 mm in diameter, however, central portions of the tumour mass were frequently either cystic, necrotic or both. Consequently, unless otherwise stated, tumours of less than 20 mm in diameter, obtained 14-21 days post inoculation (Figure 1) were used for all subsequent experiments. Haemorrhagic tumours or tumours with large necrotic or cystic areas were excluded from the study.

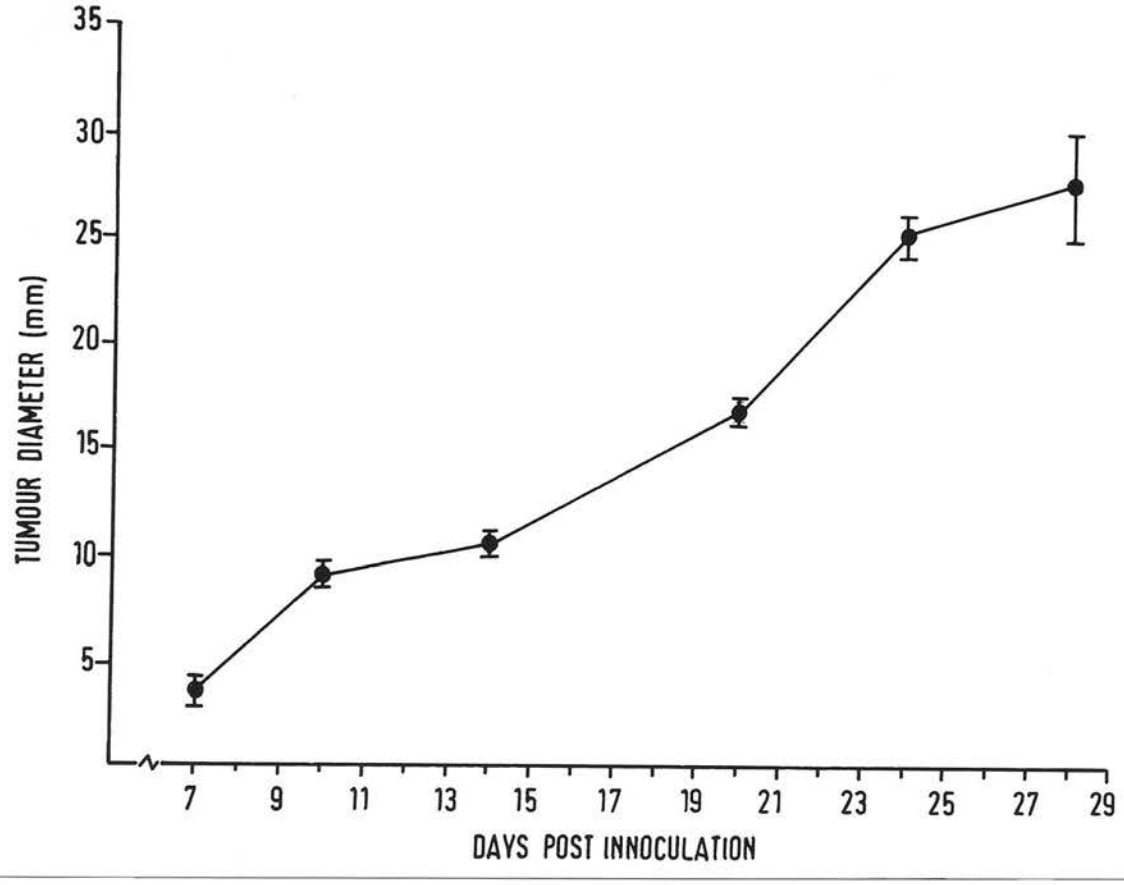

Figure 1 Growth rate of the FSA-R tumour. Animals received $5x10^5$ viable tumour cells s.c. on day 0. At intervals thereafter tumour growth was determined by taking the mean of opposing diameters. Each point represents the mean diameter $\pm$ 1 SEM of at least 8 tumours.

2.0 IDENTIFICATION AND QUANTITATION OF TUMOUR-ASSOCIATED MACROPHAGES

2.1 Identification and quantitation of macrophages in sections of tumour tissue

In this and various other tumour systems, a good correlation has generally been found between the percentage of Fc receptor-positive cells and the percentage of tumour-associated macrophages as determined by other criteria (Kerbel and Pross, 1976; Wood and Morantz, 1979; Moore and McBride, 1980, 1983). Consequently, absorption of EA to frozen sections of tumour tissue was used to determine the approximate percentage of macrophages and their distribution in the intact tumour. Roughly 25% of the surface area of tumour sections bound EA. The pattern of binding obtained could best be described as diffuse, suggesting that Fc receptor-positive cells, presumably macrophages, were distributed throughout the tumour mass.

2.2 Identification and quantitation of macrophages in tumour cell suspensions

In order to more accurately quantitate tumour-associated macrophages and isolate these cells for further study it was necessary to disaggregate the tumour tissue. Although it was considered desirable to avoid the use of enzymes, mechanical disruption always produced preparations of low yield and poor viability (< 40% excluded trypan blue). In contrast, Dispase/DNase treatment resulted in almost complete disaggregation of

tumour tissue and gave single cell suspensions of relatively high viability (> 80% excluded trypan blue).

The percentage of cells present in tumour cell suspensions which could be characterized as macrophages was determined by enumeration of both Fc receptor-positive and phagocytic cells. Fc receptor-positive cells were identified by rosette formation and phagocytic cells by uptake of latex particles. The results presented in Table I show that on average, 24.5% of the cells present in enzymatically disaggregated tumour cell suspensions expressed Fc receptors while only 20% phagocytosed latex particles. Equivalent results obtained for resident or C.parvum-elicited peritoneal cells are included for comparison. They are in agreement with published accounts of the macrophage content of these populations (Daems, 1980), and serve as a control for the reagents and procedures used.

On the basis of these results it would seem that approximately 20-25% of enzymatically disaggregated tumour cells may be classified as macrophages. This figure is very similar to the percentage of macrophages identified in tumour tissue sections by EA absorption (see section 2.1) and suggests that the enzymatic disaggregation procedure does not preferentially enrich or deplete macrophages.

TABLE I Identification and characterization of FSA-R tumour-associated macrophages.

	% Positive cells			
	Fc receptor[a]	Latex phagocytosis[b]	I-A^k[c]	MAC-1[d]
Whole resident peritoneal cells	N.D.	43.8±1.0[e]	N.D.	46.0±4.5
Adherent resident peritoneal cells	97.7±1.4	93.0±2.1	36.8±1.9	91.5±1.0
Whole *C.parvum*-elicited peritoneal cells	N.D.	45.2±1.0	N.D.	41.5±1.3
Adherent *C.parvum*-elicited peritoneal cells	98.7±0.3	92.2±1.9	N.D.	88.7±1.3
FSA-R tumour cell suspension	24.5±0.8	20.0±0.4	9.1±0.7	0.3±0.2
Adherent tumour-derived cells	96.4±1.1	92.1±1.7	56.0±1.1	4.2±0.5

a Fc receptor-positive cells were identified by EA rosette formation.

b Phagocytic cells were identified by uptake of latex particles.

c I-A^k-positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

d Mac-1-positive cells were identified by an indirect immunoperoxidase assay using the rat anti-mouse-macrophage monoclonal antibody M1/70.

e Each result represents the mean of 3 experiments ± 1 SEM.

3.0 SEPARATION OF MACROPHAGES BY ADHERENCE TO SOLID SUBSTRATES

Macrophages were enriched from tumour cell suspensions and populations of resident or C.parvum-elicited peritoneal cells by virtue of their capacity to adhere tightly to glass or plastic surfaces. Graded numbers of cells were added to tissue culture wells and incubated at 37°C for 30 minutes in the presence of 20% FCS (peritoneal cells) or 0.1% Dispase and 20% FCS (tumour cell suspensions). Non-adherent cells were removed by gentle washing and the number of adherent cells estimated by inverted phase microscopy.

For each of these cell populations an excellent linear correlation ($R > 0.99$) was found between the number of cells added to each tissue culture well and the number of cells which remained adherent (Figure 2). From the appropriate linear regression equations it can be calculated that at all cell concentrations which were examined, approximately 17.6% of tumour cells, 17.5% of resident peritoneal cells and 20.7% of C.parvum elicited peritoneal cells were adherent.

As shown in Table I more than 90% of the adherent cells isolated from tumour cell suspensions and resident or C.parvum-elicited peritoneal cells could be characterized as macrophages on the basis of Fc receptor expression and phagocytic capability.

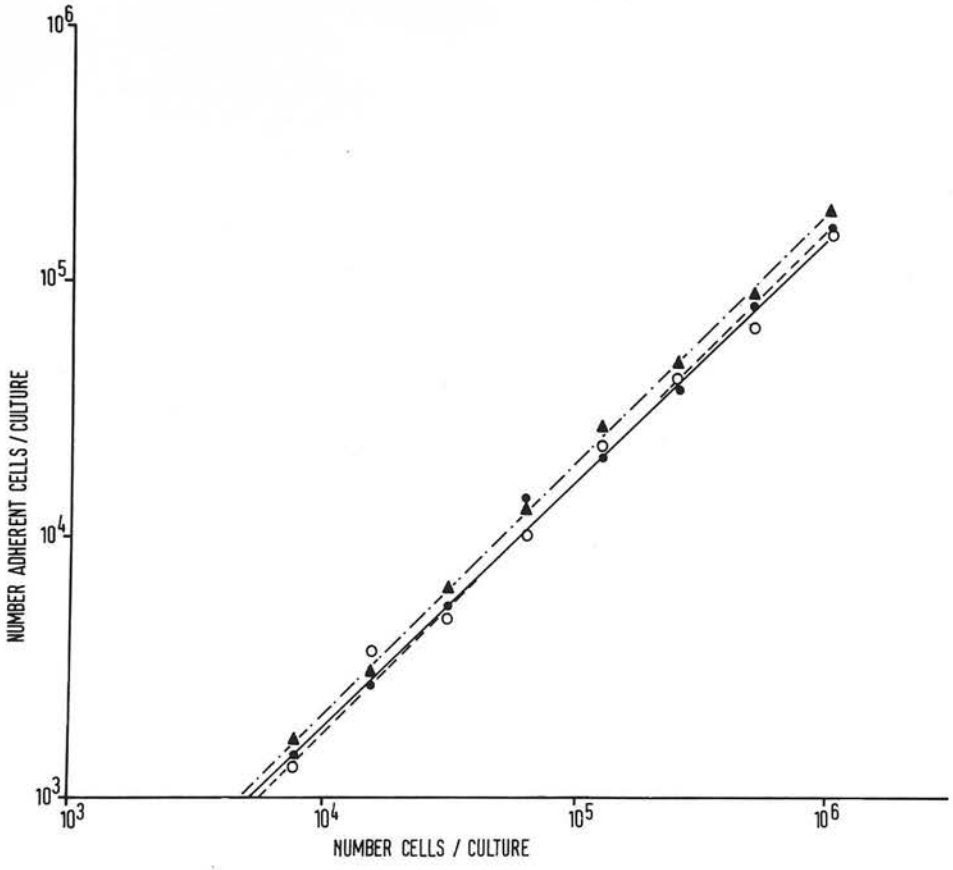

Figure 2 Quantitation of adherent macrophages by inverted phase microscopy. Various numbers of enzymatically disaggregated FSA-R tumour cells (●—●), resident peritoneal cells (○----○) or C.parvum-elicited peritoneal cells (▲-·-▲) were added to tissue culture wells and incubated at 37°C for 30 mins in presence of 20% FCS (peritoneal cells) or 0.1% Dispase and 20% FCS (tumour cell suspension). Non-adherent cells were removed by gentle washing and the adherent cells quantitated by inverted phase microscopy. For each of these cell populations an excellent linear correlation ($R > 0.99$) was found between the number of cells added to each tissue culture well and the number of cells which remained adherent.

4.0 EFFECT OF SERIAL TRANSPLANTATION ON THE MACROPHAGE CONTENT OF THE FSA-R TUMOUR

The effect of serial transplantation on the number of macrophages infiltrating FSA-R tumours was investigated. Tumours were harvested 15-20 days after inoculation of $5x10^5$ viable tumour cells and the percentage of macrophages present in enzymatically disaggregated tumour cell suspensions and monolayers of tumour-derived adherent cells was determined by EA rosette formation. As can be seen in Table II, there was no significant change in the percentage of macrophages in either of these two populations during serial transplantation of the tumour (pass 8-18).

TABLE II Effect of serial transplantation on the macrophage content of the FSA-R tumour.

Passage Number	% Fc receptor-positive cells[a]	
	Whole FSA-R	Adherent RSA-R
8	22.2 ± 1.4 [b]	92.2 ± 1.5
10	23.2 ± 1.2	92.8 ± 2.1
12	22.5 ± 1.4	93.7 ± 2.3
13	23.2 ± 0.9	96.5 ± 1.6
17	24.2 ± 0.4	97.0 ± 1.3
18	24.8 ± 0.6	93.7 ± 1.3

[a] Fc receptor-positive cells were identified by EA rosette formation.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

5.0 EFFECT OF TUMOUR GROWTH ON THE MACROPHAGE CONTENT OF THE FSA-R TUMOUR

The percentage of macrophages infiltrating the FSA-R tumour was determined as a function time. Tumours were harvested at intervals after s.c. inoculation of $5x10^5$ viable tumour cells, and the percentage of macrophages present in enzymatically disaggregated tumour cell suspensions and monolayers of tumour-derived adherent cells was determined by EA rosette formation. As indicated in Table III there was no significant change in the percentage of macrophages in either of these two populations during the observed period of tumour growth despite the finding that tumours, particularly late in growth, differed considerably with respect to size and degree of necrosis.

TABLE III Effect of tumour growth on the macrophage content of the FSA-R tumour.

Number of days postinoculation	% Fc receptor-positive cells [a] Whole FSA-R	Adherent FSA-R
7	23.3 ± 1.0[b]	94.0 ± 2.8
14	23.0 ± 1.3	93.8 ± 1.4
21	24.7 ± 1.0	94.2 ± 0.2
28	24.2 ± 1.5	95.5 ± 2.1

[a] Fc receptor-positive cells were identified by EA rosette formation.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

6.0 EXPRESSION OF MAC-1 BY TUMOUR-ASSOCIATED MACROPHAGES

Ho and Springer (1982) have proposed that the Mac-1 antigen defined by the rat anti-mouse-macrophage monoclonal antibody M1/70 (Springer et al., 1979) constitutes a universal macrophage marker. Consequently an attempt was made to use this marker to identify macrophages in tumour cell suspensions and monolayers of tumour-derived adherent cells.

As indicated in Table I an excellent correlation was found between the percentage of macrophages present in the whole or adherent populations of resident and C.parvum-elicited peritoneal cells as defined by Fc receptor expression and/or latex phagocytosis, and the percentage of Mac-1 positive cells identified using an indirect immunoperoxidase assay. In contrast, although 20-25% of enzymatically disaggregated tumour cells could be classified as macrophages on the basis of Fc receptor expression and latex phagocytosis, less than 1% of these cells expressed Mac-1. Similarly, although more than 90% of tumour-derived adherent cells were identified as macrophages on the basis of Fc receptor expression and latex phagocytosis, only around 4% of these cells expressed Mac-1.

6.1 Effect of enzyme treatment on Mac-1 expression

The possibility that the enzymatic disaggregation pro-

cedure used may have adversely affected the expression of Mac-1 by tumour-associated macrophages was investigated. It can be seen in Table IV that the enzyme treatment used to disaggregate tumour tissue and separate out macrophages from tumour cell suspensions did not significantly alter the percentage of whole or adherent resident peritoneal cells which expressed Mac-1. Thus it is not possible to attribute the very low level of Mac-1 expression on tumour-associated macrophages to the effect of the enzymatic disaggregation procedure.

6.2 Effect of in vitro culture on Mac-1 expression by tumour-associated macrophages

Tumour-derived adherent cells were cultured for various times up to 18 hours to investigate whether these cells would re-express Mac-1. The results presented in Table V show clearly that no significant change occurred in the percentage of such cells which expressed Mac-1 during 18 hours of culture.

TABLE IV Effect of enzyme treatment on Mac-1 expression by resident peritoneal cells.

Enzyme treatment	% Mac-1-positive cells [a] Whole peritoneal	Adherent peritoneal
Nil	41.7 ± 0.9 [b]	94.0 ± 1.0
0.05% Dispase/0.002% DNase 45 mins at room temp.	41.2 ± 1.6	94.3 ± 0.5
0.05% Dispase/0.002% DNase 45 mins at room temp. plus 0.1% Dispase 30 mins at 37°C.	40.9 ± 1.3	93.7 ± 0.8

[a] Mac-1-positive cells were identified by an indirect immunoperoxidase assay using the rat anti-mouse-macrophage monoclonal antibody M1/70.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

TABLE V Effect of *in vitro* culture on Mac-1 expression by tumour-associated macrophages.

Number of hours in culture	% Mac-1-positive cells [a]
0	4.2 ± 0.5 [b]
1	3.6 ± 0.8
4	3.6 ± 0.6
5	3.4 ± 0.5
18	3.7 ± 1.2

[a] Mac-1-positive cells were identified by an indirect immunoperoxidase assay using the rat anti-mouse-macrophage monoclonal antibody M1/70.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

7.0 EXPRESSION OF I-A^k BY TUMOUR-ASSOCIATED MACROPHAGES

In most experimental systems the generation of an effective immune response requires cooperation between immunocompetent lymphocytes and non-lymphoid accessory cells (reviewed by Unanue, 1981). One essential accessory cell function is to take up and process antigenic material and present the appropriate epitopes to T cells in association with Ia molecules (reviewed by Unanue et al., 1984). As a first step towards understanding the accessory cell activity of tumour-associated macrophages, the expression of Ia-determinants on these cells was investigated.

It can be seen in Table I that around 9.1% of enzymatically disaggregated tumour cells and around 56% of tumour-derived adherent cells expressed I-A^k. Although fairly heterogeneous, most of these cells appeared by morphological criteria to be "typical" macrophages (Plate 1). It is interesting to note that the I-A^k-positive cells varied considerably with respect to staining density, presumably reflecting differing levels of I-A^k expression.

7.1 Effect of enzyme treatment on I-A^k expression

The possibility that the enzymatic disaggregation procedure used may have altered the expression of I-A^k by tumour-

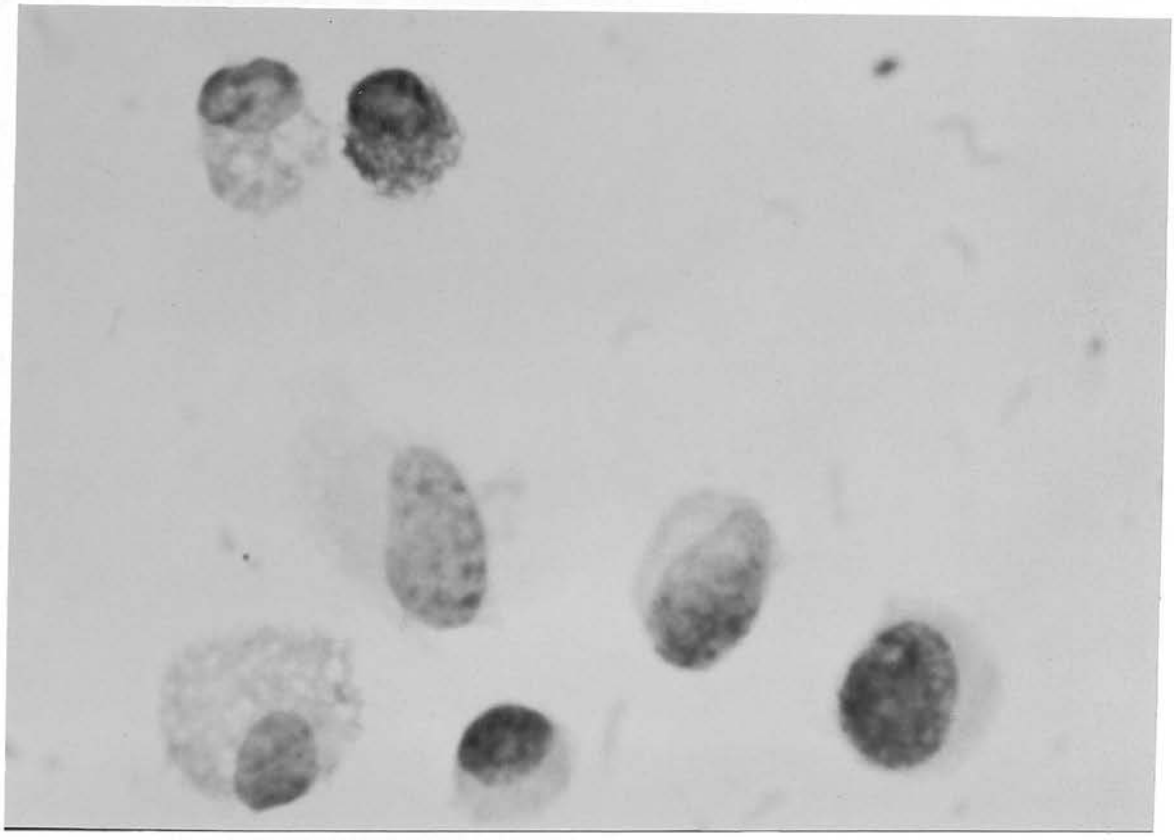

PLATE I I-A^k expression by enzymatically disaggregated tumour cells. Cytocentrifuge preparations of enzymatically disaggregated tumour cells were made and I-A^k-positive cells identified by an indirect immunoperoxidase assay using monoclonal antibody 10.2-16. (Haematoxylin counterstain, x 2000 magnification).

associated macrophages was investigated. However, it can be seen from Table VI that the enzyme treatment used to disaggregate the tumour tissue and separate macrophages out from tumour cell suspensions did not significantly alter the percentage of adherent resident peritoneal cells which expressed either I-A^k or Fc receptors.

7.2 Effect of serial transplantation of the FSA-R tumour on I-A^k expression by tumour-associated macrophages

Tumours were harvested 15-20 days after inoculation of $5x10^5$ viable tumour cells and the percentage of I-A^k-positive cells present in enzymatically disaggregated tumour cell suspensions and monolayers of tumour-derived adherent cells was determined. The results presented in Table VII show that there was no significant change in the percentage of I-A^k-positive cells in either of these two populations during serial transplantation of the tumour (pass 8-18).

7.3 Effect of tumour growth on I-A^k expression by tumour-associated macrophages

Tumours were harvested at intervals after s.c. inoculation of $5x10^5$ viable tumour cells, and the percentage of I-A^k-positive cells present in enzymatically disaggregated tumour cell suspensions and monolayers of tumour-derived adherent cells was determined. As indicated in Table VIII there was no significant change in the percentage of I-A^k

TABLE VI Effect of enzyme treatment on I-A^k and Fc receptor expression by adherent resident peritoneal cells.

Enzyme treatment	% I-A^k-positive [a]	% Fc receptor-positive [b]
Nil	35.5 ± 2.6 [c]	95.8 ± 1.5
0.05% Dispase/0.002% DNase 45 mins at room temp.	34.2 ± 1.4	95.5 ± 1.5
0.05% Dispase/0.002% DNase 45 mins at room temp plus 0.1% Dispase 30 mins at 37°C.	36.7 ± 1.7	94.3 ± 1.3

[a] I-A^k-positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

[b] Fc receptor-positive cells were identified by EA rosette formation.

[c] Each result represents the mean of 3 experiments ± 1 SEM.

TABLE VII Effect of serial transplantation on I-A^k expression by tumour-associated macrophages.

Passage number	% I-A^k-positive cells [a] Whole FSA-R	Adherent FSA-R
8	10.2 ± 0.4 [b]	48.2 ± 0.7
10	10.8 ± 0.2	50.3 ± 0.6
12	9.5 ± 0.3	49.2 ± 1.3
13	9.8 ± 0.2	50.2 ± 0.6
17	10.8 ± 0.8	50.2 ± 0.9
18	11.5 ± 0.3	51.7 ± 1.3

[a] I-A^k- positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

TABLE VIII Effect of tumour growth on I-A^k expression by tumour-associated macrophages.

Number of days postinoculation	% I-A^k-positive cells [a]	
	Whole FSA-R	Adherent FSA-R
7	10.3 ± 0.7 [b]	51.7 ± 1.7
14	10.0 ± 0.9	52.2 ± 1.4
21	10.3 ± 1.0	54.7 ± 0.7
28	9.8 ± 0.4	51.2 ± 0.6

[a] I-A^k-positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

[b] Each result represents the mean of 3 experiments ± 1 SEM.

positive cells present in either of these two populations during the observed period of tumour growth.

7.4 Regulation of I-A^k expression by tumour-associated macrophages

The effect of *in vitro* culture on I-A^k expression by tumour-associated macrophages was investigated. The results presented in Figure 3 show that I-A^k expression progressively decreased with time in culture such that after three days, the percentage of I-A^k-positive cells had fallen from approximately 50% to less than 6%. No further reduction in I-A^k expression was found if the cells were cultured for 4 days. The cells which continued to express I-A^k after 4 days in culture appeared by morphological criteria to be "typical" macrophages.

Since I-A^k expression by tumour-associated macrophages seems to be a transient event, the question arises as to what mechanisms operate *in situ* to induce and maintain I-A^k expression on such a large percentage of these cells. The involvement of T cells or their products was investigated by comparing the expression of I-A^k by macrophages obtained from tumours grown in nu/+ or nu/nu mice. The nu/nu animals lack a functional T cell system and did not reject allogeneic or xenogeneic tissue grafts. The nu/+ animals are phenotypically normal and rapidly rejected allogeneic or xenogeneic tissue grafts. The results presented in Table IX show that

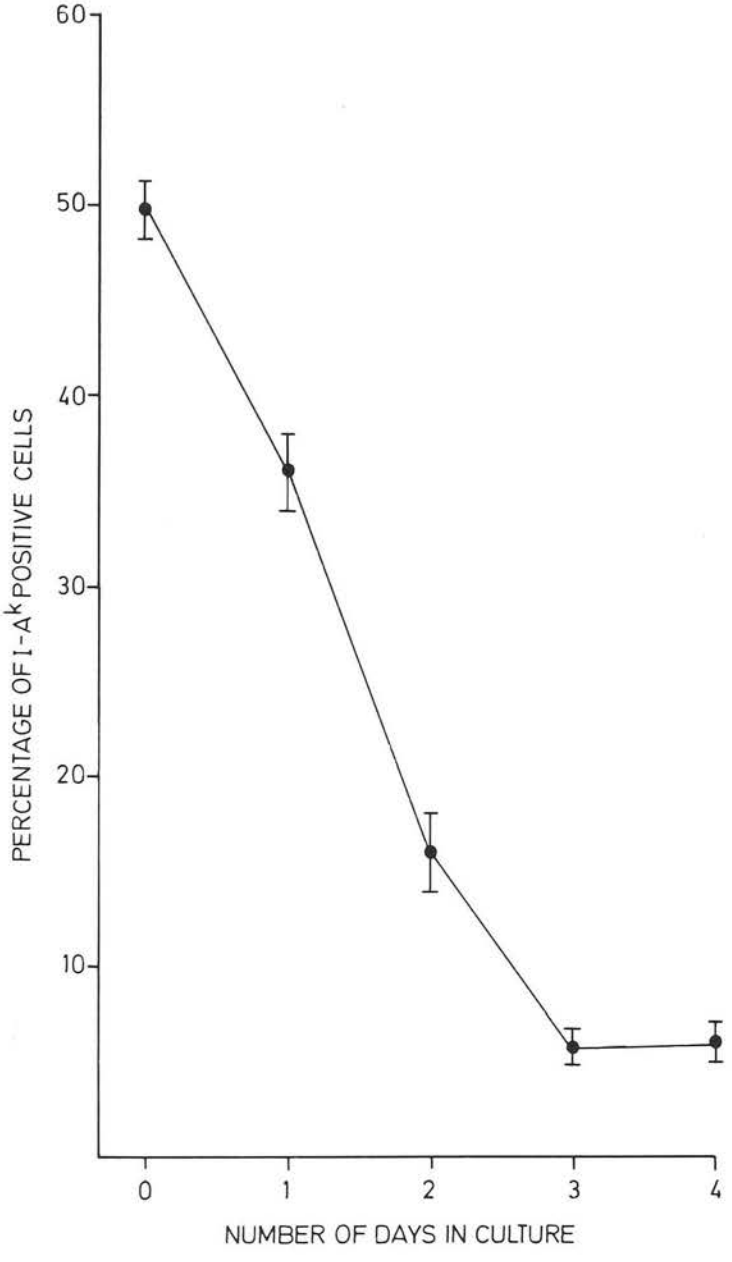

Figure 3 Effect of time in culture on I-A^k expression by tumour-associated macrophages. Adherent cells derived from $1x10^6$ viable enzymatically disaggregated tumour cells were cultured for various periods of time, fixed and the percentage of I-A^k-positive cells determined by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16. Each point represents the mean of 3 experiments $\pm$ 1 SEM.

TABLE IX $I\text{-}A^k$ expression by macrophages obtained tumours grown in nu/+ or nu/nu mice.

Tumour-bearing mice	% Fc receptor-positive[a] Whole FSA-R	Adherent FSA-R	% $I\text{-}A^k$-positive[b] Whole FSA-R	Adherent FSA-R
nu/+	24.0 ± 0.8[c]	94.0 ± 0.8	11.1 ± 0.6	50.2 ± 0.9
nu/nu	24.2 ± 0.6	93.7 ± 0.6	1.1 ± 0.2	0.8 ± 0.2

a Fc receptor-positive cells were identified by EA rosette formation.

b $I\text{-}A^k$-positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

c Each result represents the mean of 3 experiments $\pm$1 SEM.

enzymatically disaggregated tumour cell suspensions prepared from FSA-R tumours grown in nu/+ or nu/nu mice did not differ significantly in their content of Fc receptor-positive cells. However, while around 11.1% of enzymatically disaggregated tumour cells derived from tumours grown in nu/+ animals expressed I-A^k only 1.1% of cells derived from tumours grown in nu/nu animals expressed the antigen. Similarly, 50.2% of adherent cells derived from tumours grown in nu/+ animals were I-A^k positive, but only 0.8% of adherent cells from tumours grown in nu/nu animals were positive.

These results suggest that T cells or their products constitute a major regulatory mechanism which determines I-A^k expression by tumour-associated macrophages.

SECTION II - ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES. I. GENERATION OF A PRIMARY PFC RESPONSE IN VITRO.

1.0 DETERMINATION OF OPTIMAL CULTURE TIME AND DOSE OF ANTIGEN FOR THE GENERATION OF A PRIMARY ANTI-CRBC PFC RESPONSE IN VITRO

PFC responses were generated in Nunc 24 well tissue culture plates (Gibco Bio-Cult Ltd., Paisley, Scotland.). 5×10^6 whole normal spleen cells were cultured with various numbers of CRBC in 2 ml of RPMI + 10% FCS. The magnitude of the anti-CRBC PFC response generated depended on both the time in culture and the dose of antigen used (Table X). Maximal responses were consistantly obtained in cultures harvested on day 4 which received 10^7 CRBC. Consequently, these same culture conditions and the optimal culture time and antigen dose were used for all subsequent experiments.

TABLE X Determination of optimal culture time and dose of antigen for the generation of a primary anti-CRBC PFC response *in vitro*.[a]

Number CRBC culture	Number anti-CRBC PFC/culture Day 2	Day 3	Day 4	Day 5
Nil	33 ± 7[b]	33 ± 9	23 ± 9	20 ± 6
10^5	47 ± 12	50 ± 11	123 ± 15	47 ± 9
10^6	33 ± 9	117 ± 32	270 ± 17	150 ± 25
10^7	60 ± 12	287 ± 29	613 ± 14	403 ± 12
10^8	53 ± 12	310 ± 51	500 ± 15	420 ± 17

a 5×10^6 whole normal spleen cells were incubated with various numbers of CRBC and the magnitude of the anti-CRBC PFC response generated was determined on day 2 to day 5 of culture.

b Each result represents the mean ± 1 SEM of triplicate cultures.

2.0 ROLE OF ACCESSORY CELLS IN THE GENERATION OF A PRIMARY ANTI-CRBC PFC RESPONSE IN VITRO

Normal spleen cells were depleted of accessory cells by passage through a Sephadex G-10 column (Ly and Mishell, 1974). Approximately 35-40% of those cells initially applied to the column were recovered following this procedure. Analysis of cell surface markers by indirect immunoperoxidase (Table XI) revealed that passage through Sephadex G-10 reduced the percentage macrophages (Mac-1-positive cells) present in the cell suspension from 10% to less than 0.5%. In contrast, the ratio of T lymphocytes (Thy-1-positive cells) to B lymphocytes (Ig-positive cells) in the effluent population was approximately the same as found in normal spleen cell suspensions.

Passage of normal spleen cells through Sephadex G-10 also greatly reduced the ability of these cells to generate a primary anti-CRBC PFC response in vitro (Figure 4). In order to ensure that such depletion was the result of the removal of accessory cells and not some other cell type, it was necessary to show that the depleted response could be reconstituted by the addition of accessory cells. The results presented in Figure 4 show that the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells could be reconstituted in a dose-dependent fashion by the addition of adherent resident peritoneal cells. Maximum reconstitution was

TABLE XI Characterization of whole and Sephadex G-10 passed spleen cell suspensions

	% Positive Cells [a]		
	Mac-1	Thy-1.2	Ig
Whole	10.1 ± 3.6	22.3 ± 2.6	58.3 ± 4.2
Sephadex G-10 passed	0.4 ± 0.1	28.8 ± 3.5	69.6 ± 5.1

[a] Determined on fixed cytocentrifuge preparations by immunoperoxidase

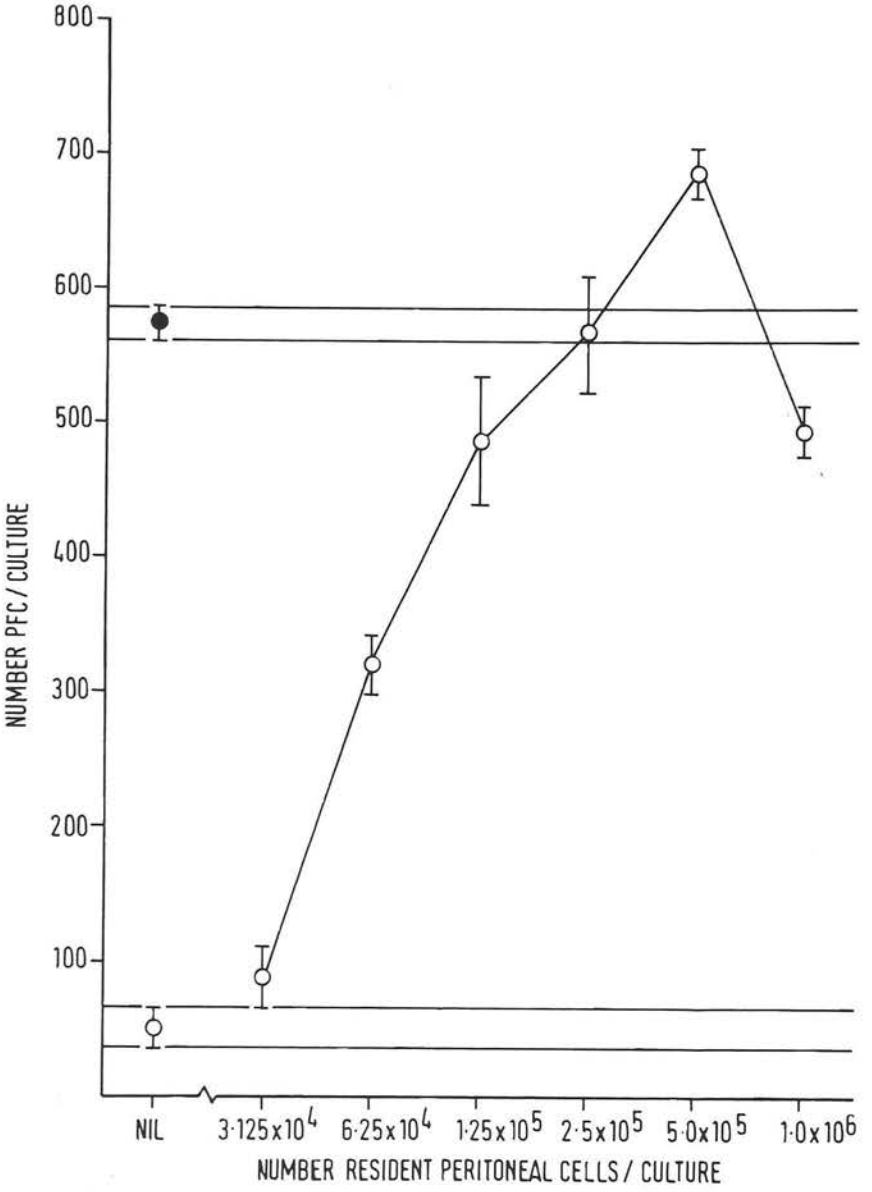

Figure 4 Reconstitution of the primary anti-CRBC response of Sephadex G-10-passed spleen cells by adherent resident peritoneal cells. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex-G10 passed spleen cells and varying numbers of resident peritoneal cells (○) were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was determined on day 4. Each point represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 70 anti-CRBC PFC/culture.

obtained with adherent cells from $5x10^5$ resident peritoneal cells. This cell number gives monolayers containing approximately $8.75x10^4$ adherent cells (Figure 2) more than 90% of which may be classified as macrophages on the basis of Fc receptor expression, latex phagocytosis and Mac-1 expression (Table 1). Thus optimal responses were obtained in cultures containing 1-2% macrophages. Exceeding this number resulted in a reduction in the magnitude of the response generated.

3.0 ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES

The ability of adherent cells isolated from the FSA-R tumour to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells was investigated. The results presented in Figure 5 demonstrate that the tumour-derived adherent cells were fully capable of reconstituting the depleted response. The degreeof reconstitution obtained depended on the number of adherent cells added. Maximum reconstruction was obtained in cultures which received adherent cells from $5x10^5$ enzymatically disaggregated tumour cells. This cell number gives monolayers containing approximately $8.8x10^4$ adherent cells (Figure 2), 90% of which may be classified as macrophages more than on the basis of Fc receptor expression and latex phagocytosis (Table I). Thus as with resident peritoneal cells, optimal responses were obtained in cultures which contained 1 - 2% tumour-associated macrophages. Exceeding this number resulted in a considerable reduction in the magnitude of the response generated.

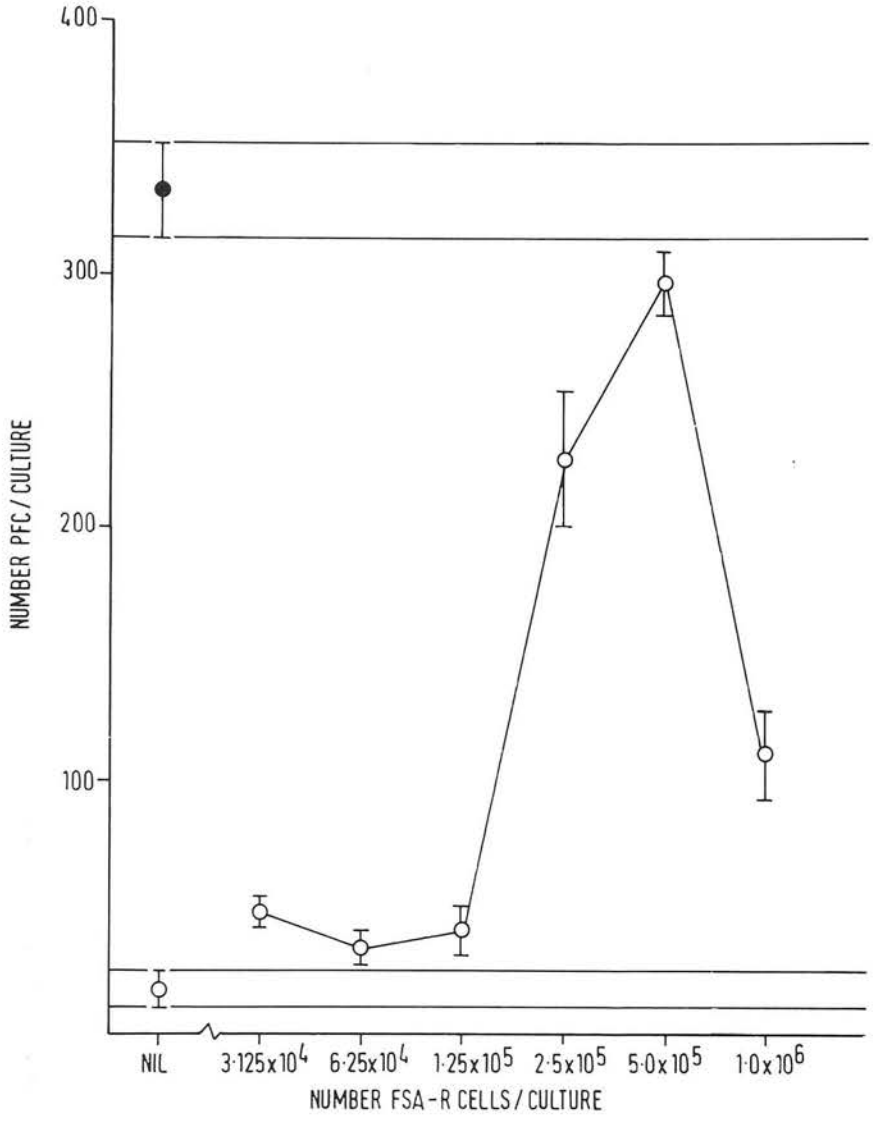

Figure 5 Reconstitution of the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells by tumour-derived adherent cells. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells and various numbers of tumour-derived adherent cells (○) were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was determined on day 4. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 70 anti-CRBC PFC/culture.

4.0 CHARACTERIZATION OF THE ACCESSORY CELLS PRESENT IN THE TUMOUR-ASSOCIATED MACROPHAGE POPULATION

4.1 Effect of anti-Ia serum and complement treatment on the accessory cell activity of tumour-associated macrophages

Monolayers of adherent tumour-associated macrophages were depleted of cells expressing Ia antigens by treatment with anti-Ia serum plus complement. This procedure resulted in the loss of approximately 40% of the macrophages from the monolayers and less than 5% of the remaining cells were found to express I-A^k determinants using an indirect immunoperoxidase assay (Table XII). The ability of untreated and anti-Ia serum plus complement treated tumour-associated macrophages to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells was investigated. The results presented in Figure 6 show clearly that treatment of tumour-associated macrophages with anti-Ia serum and complement effectively abolished the ability of these cells to reconstitute the depleted response. The use of various macrophage doses rules out the possibility that this finding was the result of an inappropriate number of macrophages remaining in the tissue culture well after anti-Ia serum plus complement treatment. Instead, it appears that Ia-positive cells are required for the expression of accessory cell activity by tumour-associated macrophages.

TABLE XII Effect of anti-Ia serum plus complement treatment on I-A^k expression by tumour-derived adherent cells

	% I-A^k positive cells [a]	% cells recovered [b]
Untreated	52.0±1.7	100
Guinea pig serum (1/20) 45 mins at 37°C	51.0±2.5	92
ATH anti-ATL_o serum (1/20) 30 mins at 0°C plus Guinea pig serum (1/20) 45 mins at 37°C	4.0±0.6	59

a I-A^k-positive cells were identified by an indirect immunoperoxidase assay using monoclonal antibody 10-2.16.

b The percentage of cells remaining adherent after treatment was determined by inverted phase microscopy.

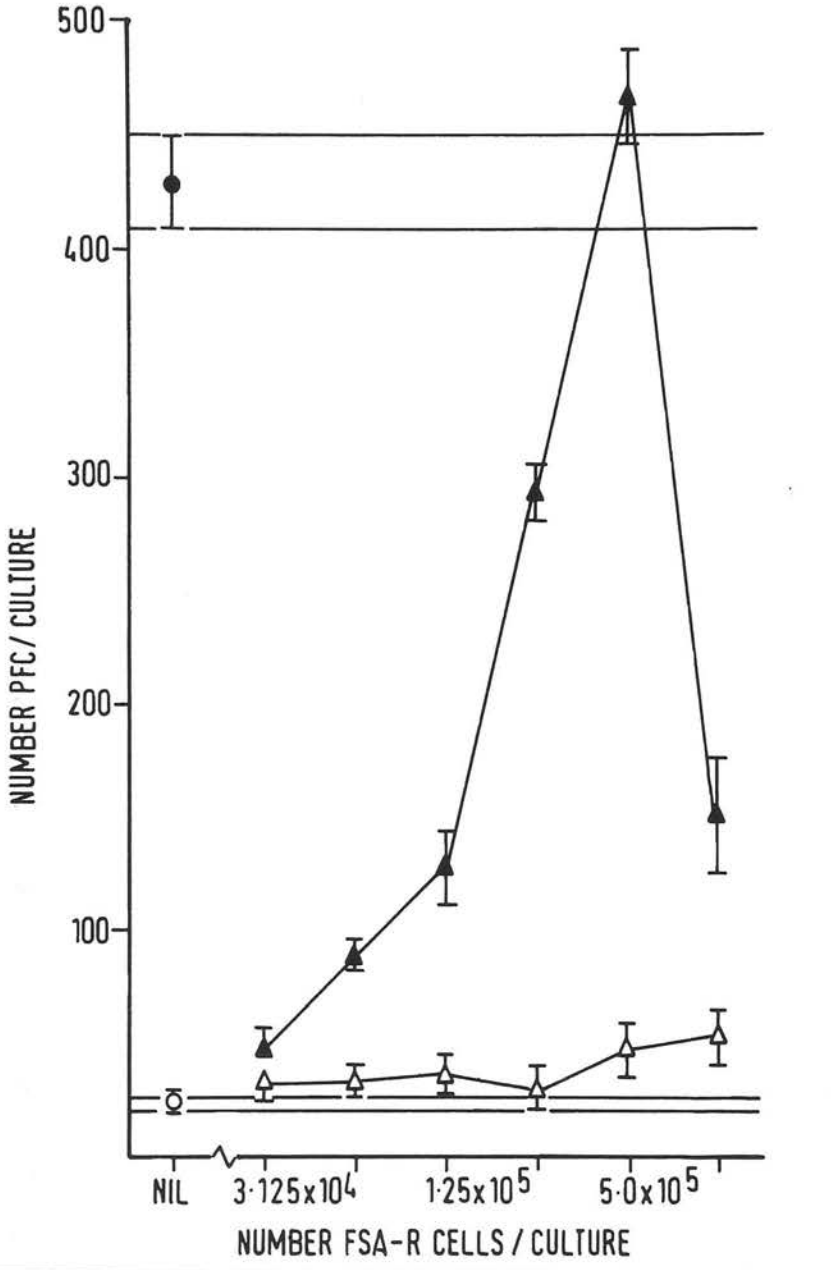

Figure 6 Effect of anti-Ia serum plus complement treatment on the ability of tumour-derived adherent cells to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells (○) were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was assayed on day 4. The effect of the addition of various numbers of untreated (▲) or anti-Ia serum plus complement treated (△) tumour-derived adherent cells on the response of Sephadex G-10 passed spleen cells was determined. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 30 anti-CRBC PFC/culture.

4.2 Comparison of the accessory cell activity of macrophages obtained from tumours grown in nu/+ or nu/nu mice

Macrophages obtained from tumours grown in nu/+ animals were approximately 50% I-A^k positive (Table IX) and were able to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells (Figure 7). In contrast, macrophages obtained from tumours grown in nu/nu animals did not express detectable I-A^k (Table IX) and were unable to reconstitute the Sephadex G-10 depleted response (Figure 7). These results support the view that Ia-positive cells are required for the expression of accessory cell activity by tumour-associated macrophages.

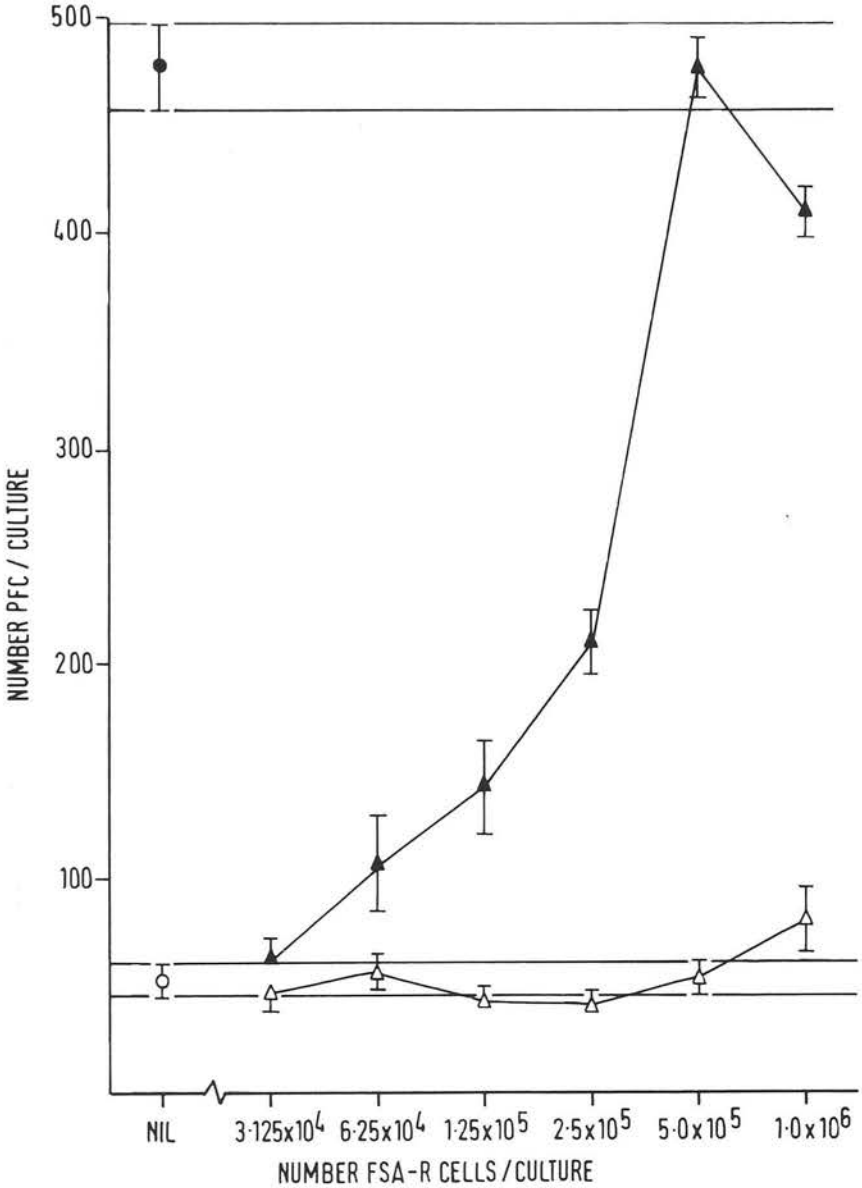

Figure 7 Reconstitution of the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells by adherent cells derived from tumours grown in nu/+ or nu/nu mice. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells (○) were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was determined on day 4. The effect of the addition of various numbers of adherent cells derived from tumours grown in nu/+ (▲) or nu/nu (△) mice on the response of Sephadex G-10 passed spleen cells was determined. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 70 anti-CRBC PFC/culture.

SECTION III ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES. II. COOPERATION BETWEEN T_H CELLS AND B CELLS IN THE GENERATION OF A PFC RESPONSE IN VITRO

1.0 EFFECT OF NYLON WOOL-PASSED CRBC-PRIMED SPLEEN CELLS ON THE ANTI-CRBC PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS

One of the most important factors limiting the number of PFC generated in cultures of whole normal spleen cells and antigen is the number of T_H cells that can recognise and respond to the antigen (Gisler et al., 1981). Previous studies have shown that nylon wool-passed spleen cells from animals primed against CRBC constitute an excellent source of CRBC-specific T_H cells (Howie and McBride, 1982b). As indicated in Figure 8 the addition of these cells to cultures of whole normal spleen cells and CRBC increased considerably the magnitude of the anti-CRBC PFC response generated. Initially, this increase depended on the number of nylon wool-passed spleen cells added. However no further increase was observed if more than 5×10^5 cells were added. Presumably at this point T_H is now in excess and it is the number of responding B lymphocytes or accessory cells that limit the magnitude of the response generated. If nylon wool-passed spleen cells from normal unprimed mice were added to cultures of whole normal spleen and CRBC, there was no significant increase in the anti-CRBC PFC response generated. Presumably this population does not contain sufficient CRBC-specific T_H cells.

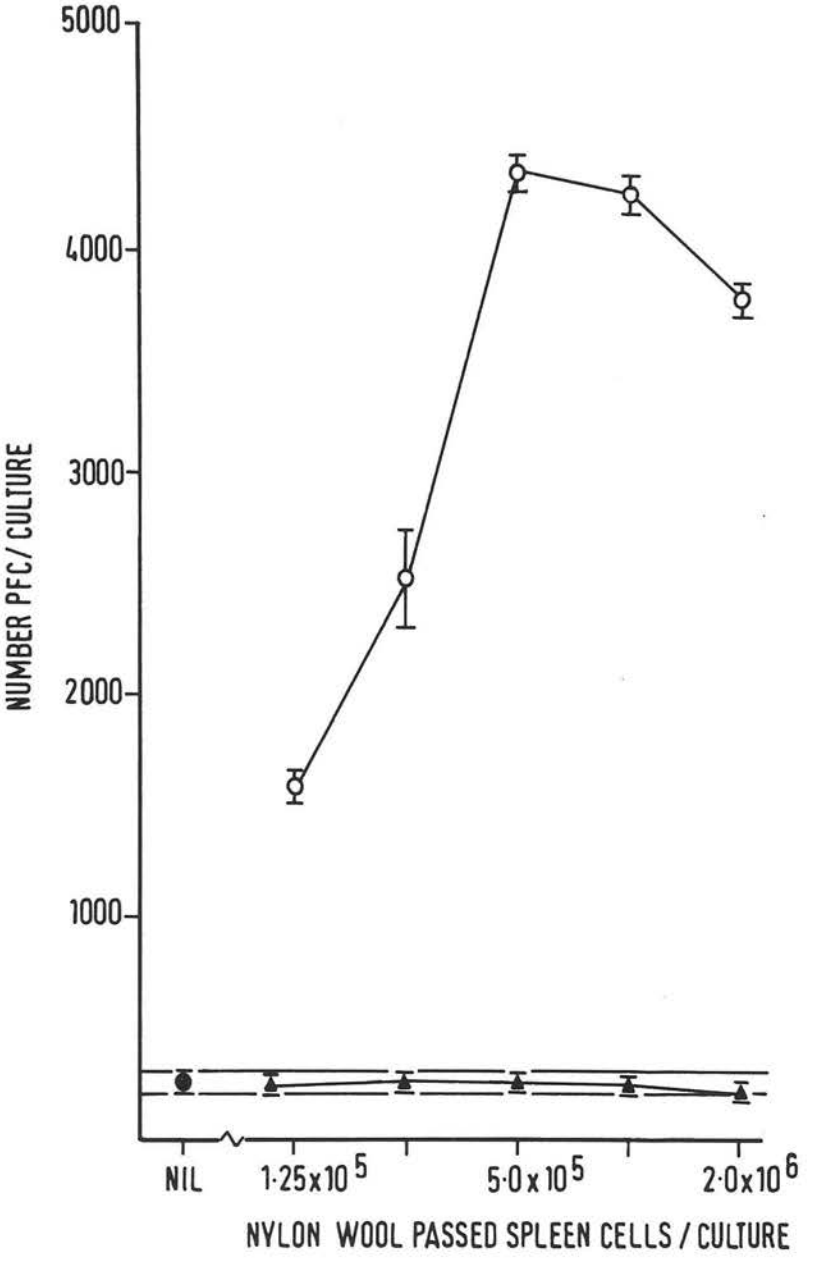

Figure 8 Effect of nylon wool-passed CRBC-primed spleen cells on the anti-CRBC PFC response of whole normal spleen cells. 5 x 10^6 whole normal spleen cells were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was assayed on day 4 (●). The effect of the addition of various numbers of nylon wool-passed CRBC-primed (○) or unprimed (▲) spleen cells on response generated was determined. Each point represents the mean ± 1 SEM of triplicate cultures.

2.0 ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES IN THE GENERATION OF AN ANTI-CRBC PFC RESPONSE IN THE PRESENCE OF EXCESS T_H CELLS

Cultures containing 5 x 10^6 whole normal spleen cells, 1 x 10^6 nylon wool-passed CRBC-primed spleen cells and 10^7 CRBC, generated a large anti-CRBC PFC response (Figure 9). If the responding normal spleen cells were passed through a Sephadex G-10 column then the response generated was greatly reduced (Figure 9). However the Sephadex G-10 depleted response could be reconstituted in a dose-dependent fashion by the addition of tumour-derived adherent cells (Figure 9). These results suggest that cooperation between antigen-primed T_H cells and normal B cells in the generation of an antibody response *in vitro* requires the presence of accessory cells and that tumour-associated macrophages may provide this accessory cell function.

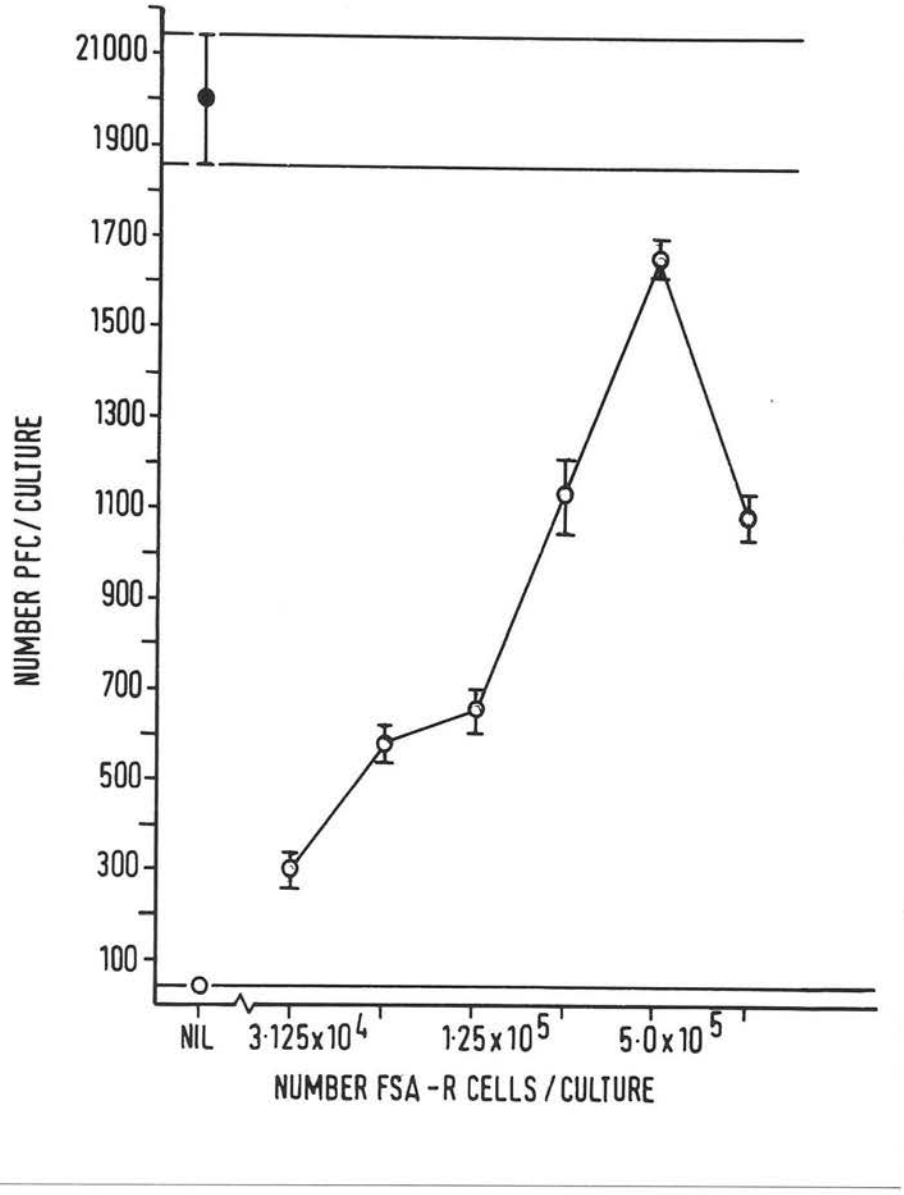

Figure 9 Accessory cell activity of tumour-associated macrophages in cooperation between CRBC-primed T_H cells and normal B cells in the generation of an anti-CRBC PFC response. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells and various numbers of tumour-derived adherent cells (○) were incubated with 1×10^6 nylon wool-passed CRBC-primed spleen cells and 10^7 CRBC. The anti-CRBC PFC response generated was assayed on day 4. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 150 anti-CRBC PFC/culture.

3.0 EFFECT OF NYLON WOOL-PASSED CARRIER-PRIMED SPLEEN CELLS ON THE ANTI-HAPTEN PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS

Cultures containing 5 x 10^6 whole normal spleen cells and 10^7 TNP-CRBC generated a small anti-TNP PFC response (< 400 PFC/culture) (Figure 10). However this response was enhanced in a dose-dependent fashion by the addition of nylon wool-passed spleen cells from animals primed against the CRBC carrier (Figure 10). Maximum enhancement was obtained in cultures which received 5 x 10^5 or more such cells. Addition of nylon wool-passed spleen cells from unprimed animals produced no increase in the anti-TNP PFC response generated. These results indicate that carrier-primed T cells can cooperate with normal B cells to generate an anti-hapten PFC response in the presence of hapten-carrier conjugates.

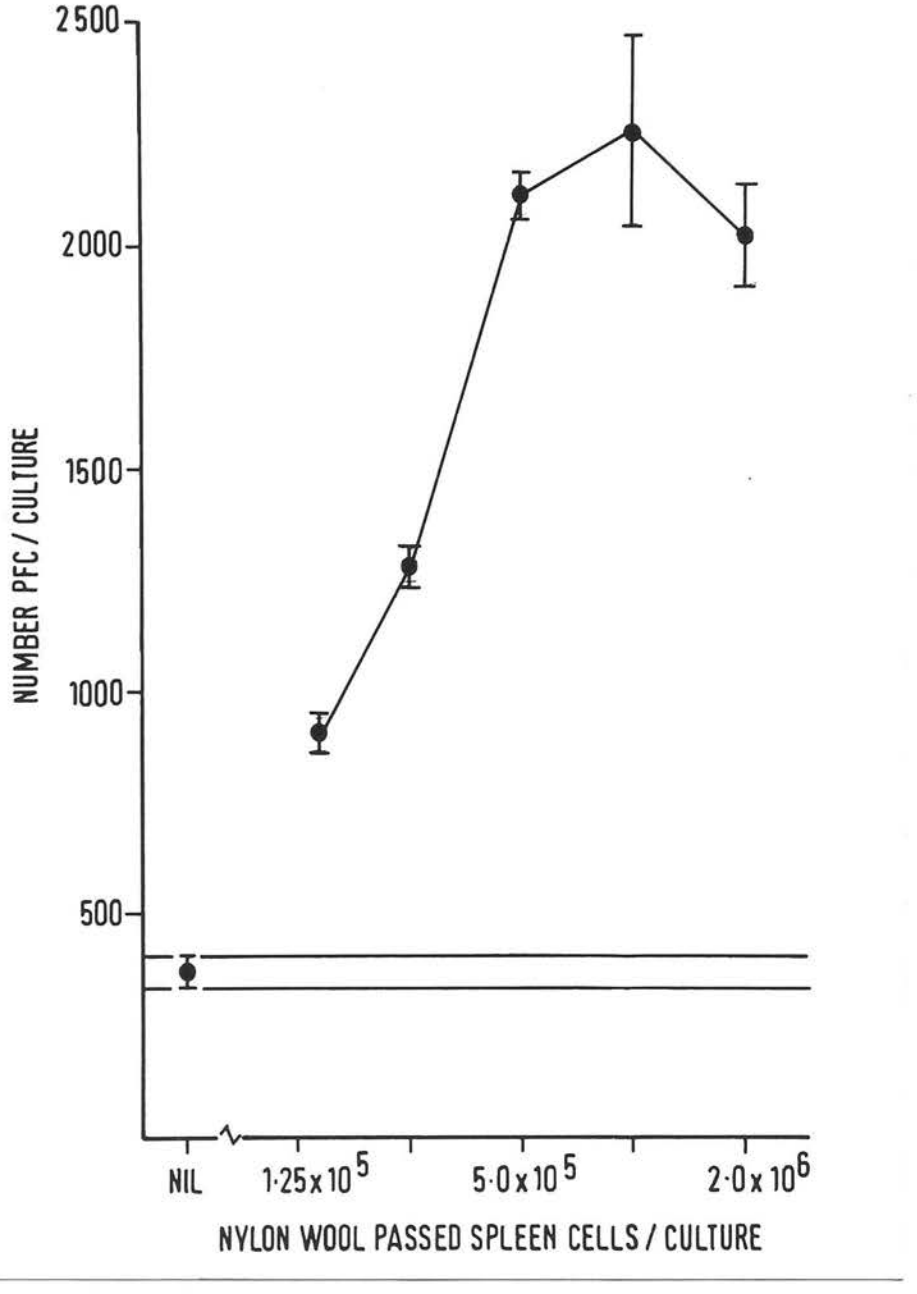

Figure 10 Effect of nylon wool-passed carrier-primed spleen cells on the anti-hapten PFC response of whole normal spleen cells. 5×10^6 whole normal spleen cells and various numbers of nylon wool-passed CRBC-primed spleen cells were incubated with 10^7 TNP-CRBC and the anti-TNP PFC response generated was assayed on day 4. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive TNP-CRBC always generated less than 250 anti-TNP PFC/culture.

4.0 MACROPHAGE ACCESSORY CELL ACTIVITY IN THE GENERATION OF AN ANTI-HAPTEN PFC RESPONSE IN THE PRESENCE OF EXCESS CARRIER-SPECIFIC T_H CELLS

Cultures containing 5 x 10^6 whole normal spleen cells, 1 x 10^6 nylon wool-passed CRBC-primed spleen cells and 10^7 TNP-CRBC generated a large anti-TNP PFC response (Figures 11 and 12). Passage of the responding normal spleen cells through Sephadex G-10 greatly reduced the magnitude of the response generated (Figures 11 and 12). However, this depleted response could be reconstituted in a dose dependent fashion by the addition of adherent resident peritoneal cells (Figure 11) or adherent tumour-derived cells (Figure 12). These results suggest that cooperation between carrier-primed T_H cells and B cells in the generation of an anti-hapten PFC response in vitro requires the presence of accessory cells, and that both resident peritoneal macrophages and tumour-associated macrophages can provide this accessory cell function.

Howie and McBride (1982b)have demonstrated the presence of significant numbers of tumour-specific T_H cells in the spleens of animals bearing FSA-R tumours. Experiments were performed to evaluate the accessory cell activity of tumour-associated macrophages in cooperation between tumour-specific T_H cells and normal B cells. 5 x 10^6 whole normal spleen cells incubated with 1 x 10^6 nylon wool-passed spleen cells from a day 14 tumour bearer and 10^5 TNP-FSA-R generated a large anti-TNP PFC response

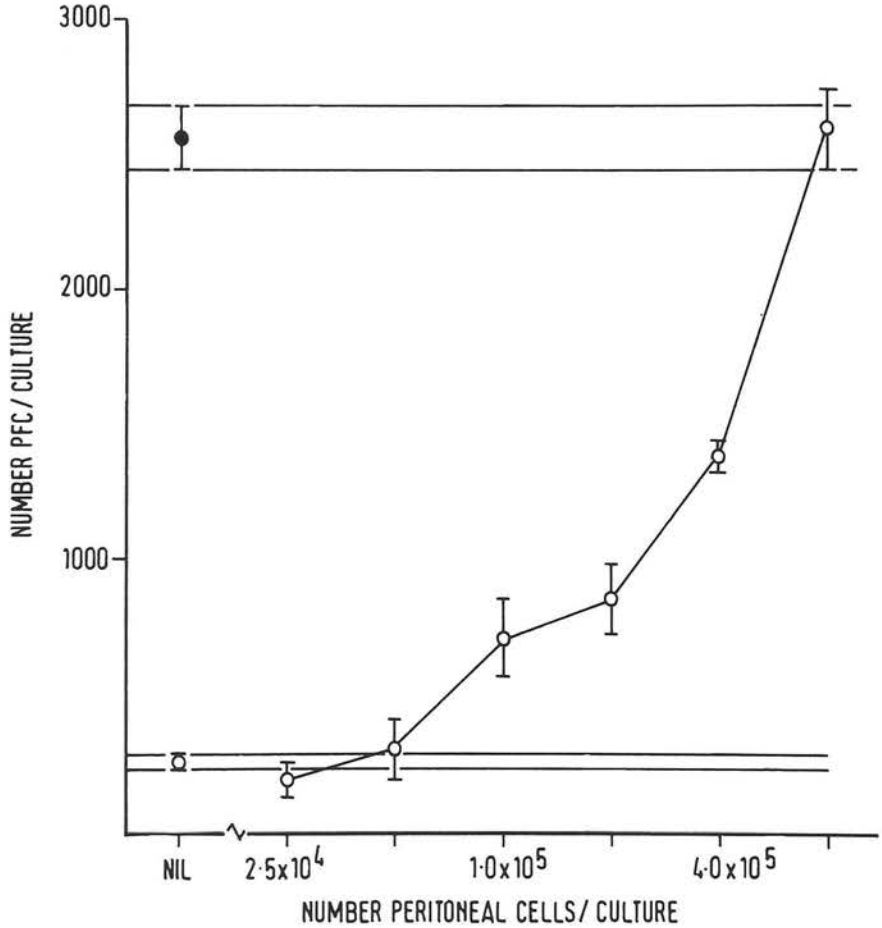

Figure 11 Accessory cell activity of resident peritoneal macrophages in cooperation between CRBC-primed T_H cells and normal B cells in the generation of an anti-TNP PFC response in the presence of TNP-CRBC. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed normal spleen cells and various numbers of adherent resident peritoneal cells (○) were incubated with 1×10^6 nylon wool-passed CRBC-primed spleen cells and 10^7 TNP-CRBC and the anti-TNP PFC response generated was assayed on day 4. Each point represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive TNP-CRBC generated less than 100 anti-TNP PFC/culture.

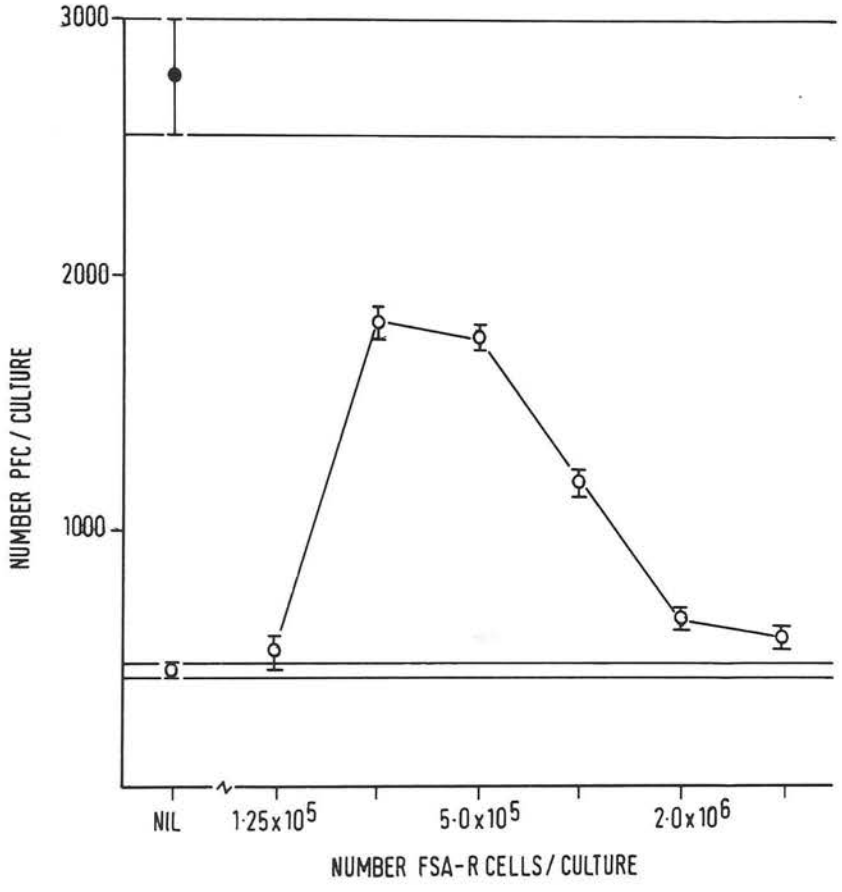

Figure 12 Accessory cell activity of tumour-associated macrophages in cooperation between CRBC-primed T_H cells and normal B cells in the generation of an anti-TNP PFC response in the presence of TNP-CRBC. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells and various numbers of tumour-derived adherent cells (○) were incubated with 1.7×10^6 nylon wool-passed CRBC-primed spleen cells and 10^7 TNP-CRBC, and the anti-TNP PFC response generated was assayed on day 4. Each point represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive TNP-CRBC generated less than 100 anti-TNP PFC/culture.

(Figure 13). This response was dependent upon the presence of accessory cells and was greatly reduced if the responding normal spleen cells were passed through Sephadex G-10 (Figure 13). The Sephadex G-10 depleted response could however be reconstituted in a dose-dependent fashion by the addition of tumour-derived adherent cells (Figure 13).

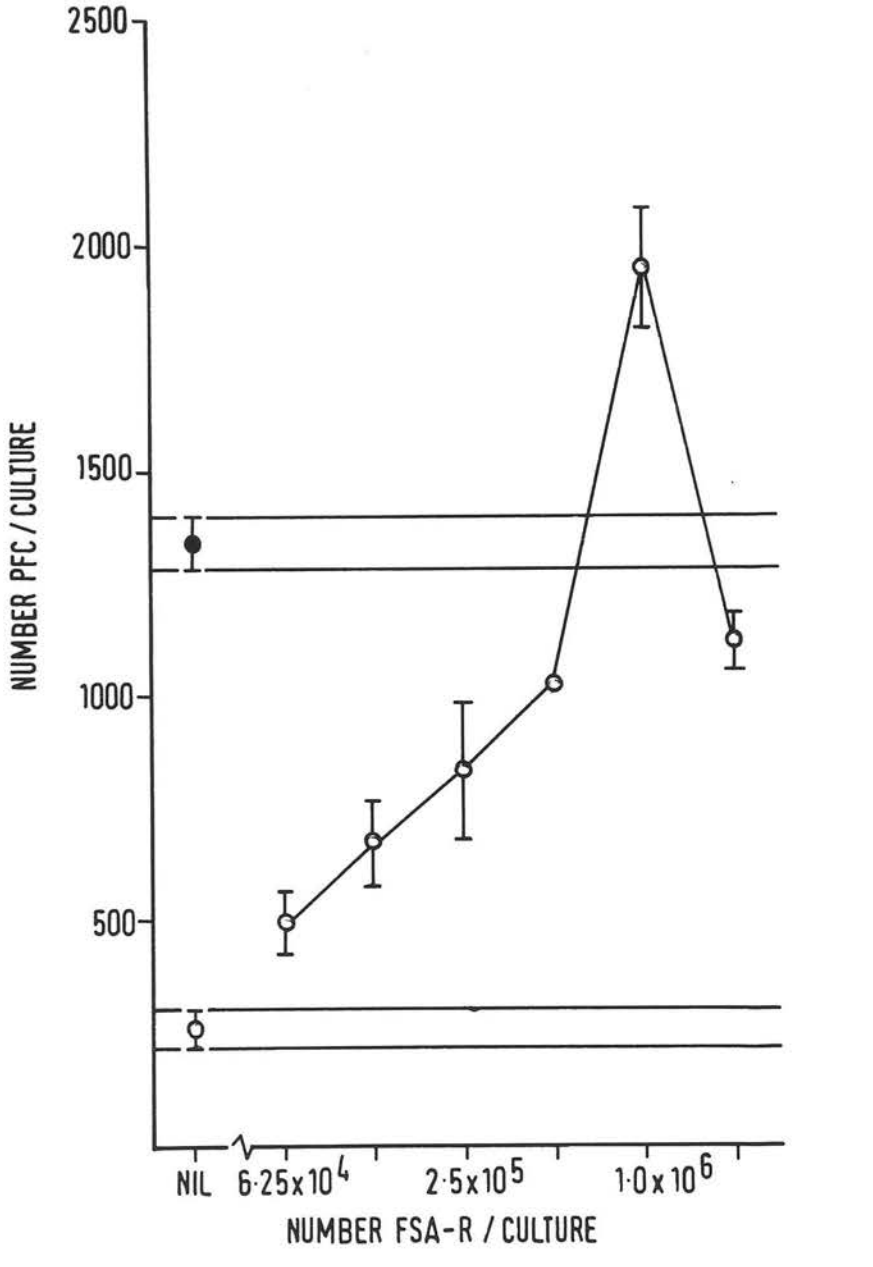

Figure 13. Accessory cell activity of tumour-associated macrophages in cooperation between tumour-specific T_H cells and normal B cells in the generation of an anti-TNP PFC response in the presence of TNP-FSA-R. 5×10^6 whole normal spleen cells (●) or 5×10^6 Sephadex G-10 passed spleen cells and various numbers of tumour-derived adherent cells (○) were incubated with 1×10^6 nylon wool-passed spleen cells from an animal bearing a day 14 FSA-R tumour and 10^5 TNP-FSA-R. The anti-TNP PFC response generated was assayed on day 4. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Cultures which did not receive TNP-FSA-R generated less than 100 anti-TNP PFC/culture.

SECTION IV ACCESSORY CELL ACTIVITY OF TUMOUR-ASSOCIATED MACROPHAGES. III. ENHANCEMENT OF THE PRIMARY PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS

1.0 EFFECT OF RESIDENT PERITONEAL MACROPHAGES ON THE PRIMARY ANTI-CRBC PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS

In order to evaluate macrophage suppressor cell activity, graded numbers of adherent resident peritoneal cells were added to cultures containing 5×10^6 whole normal spleen cells and 10^7 CRBC, and their effect on the anti-CRBC PFC response generated was determined. It was found that even at the highest concentrations examined, adherent resident peritoneal cells did not significantly alter the anti-CRBC PFC response, (Figure 14). Furthermore, treatment of adherent resident peritoneal cells with the enzyme procedure used to disaggregate tumour tissue and separate out tumour-associated macrophages did not alter their activity in this assay system (Figure 14).

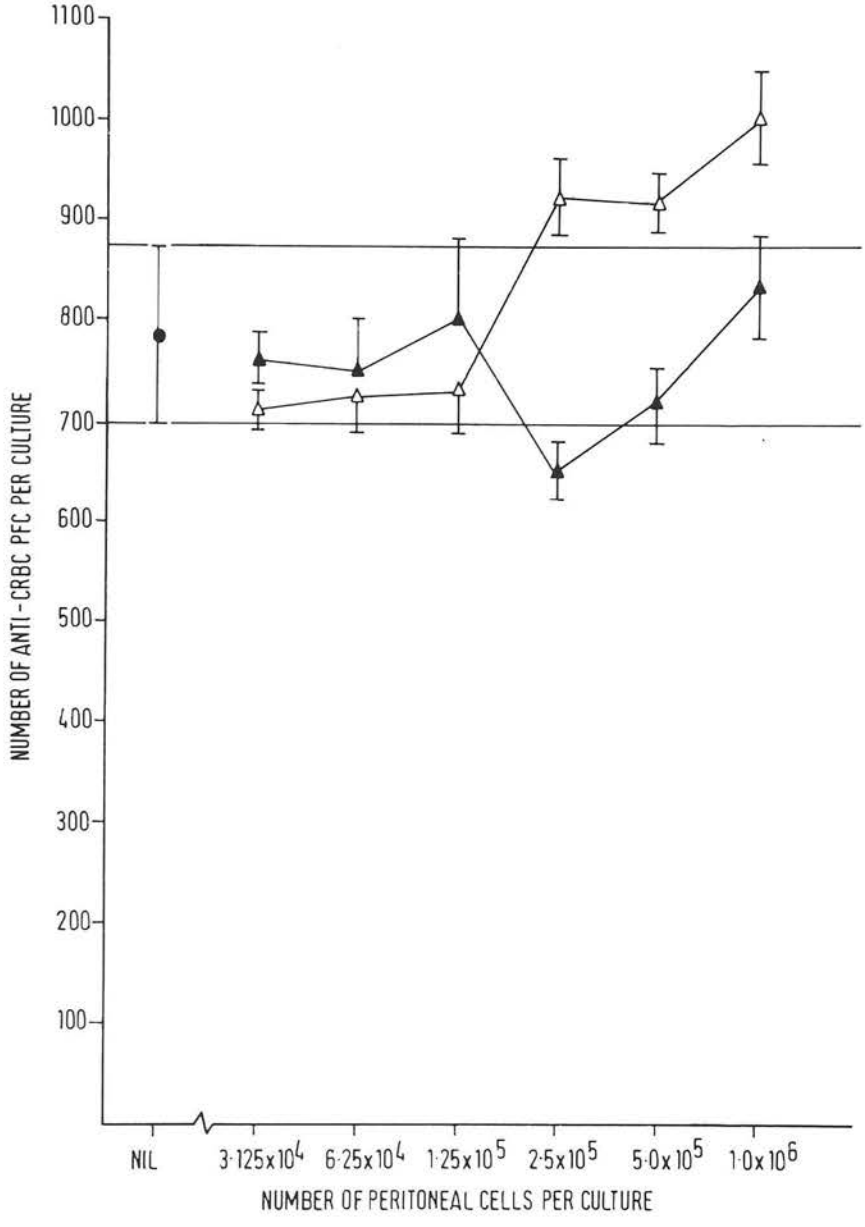

Figure 14 Effect of untreated and enzyme treated resident peritoneal macrophages on the primary anti-CRBC PFC response of whole normal spleen cells. $5_7 \times 10^6$ whole normal spleen cells were incubated with 10^7 CRBC and the primary anti-CRBC PFC response generated was assayed on day 4 (●). The effect of addition of various numbers of untreated adherent resident peritoneal cells (△) or adherent resident peritoneal cells which had been treated with 0.05% Dispase/0.002% DNase for 45 minutes at room temperature plus 0.1% Dispase for 30 minutes at 37°C (▲), was determined. Each point represents the mean ± 1 SEM of triplicate cultures Equivalent cultures which did not receive CRBC generated less than 50 anti-CRBC PFC/culture.

2.0 EFFECT OF TUMOUR-ASSOCIATED MACROPHAGES ON THE PRIMARY ANTI-CRBC PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS

Graded numbers of tumour-derived adherent cells were added to cultures containing 5×10^6 whole normal spleen cells and 10^7 CRBC and their effect on the primary anti-CRBC PFC response generated was determined. It is evident from the results presented in Figure 15, that instead of suppressing the anti-CRBC PFC response as expected, tumour-associated macrophages actually caused a dose-dependent increase in the magnitude of the response generated.

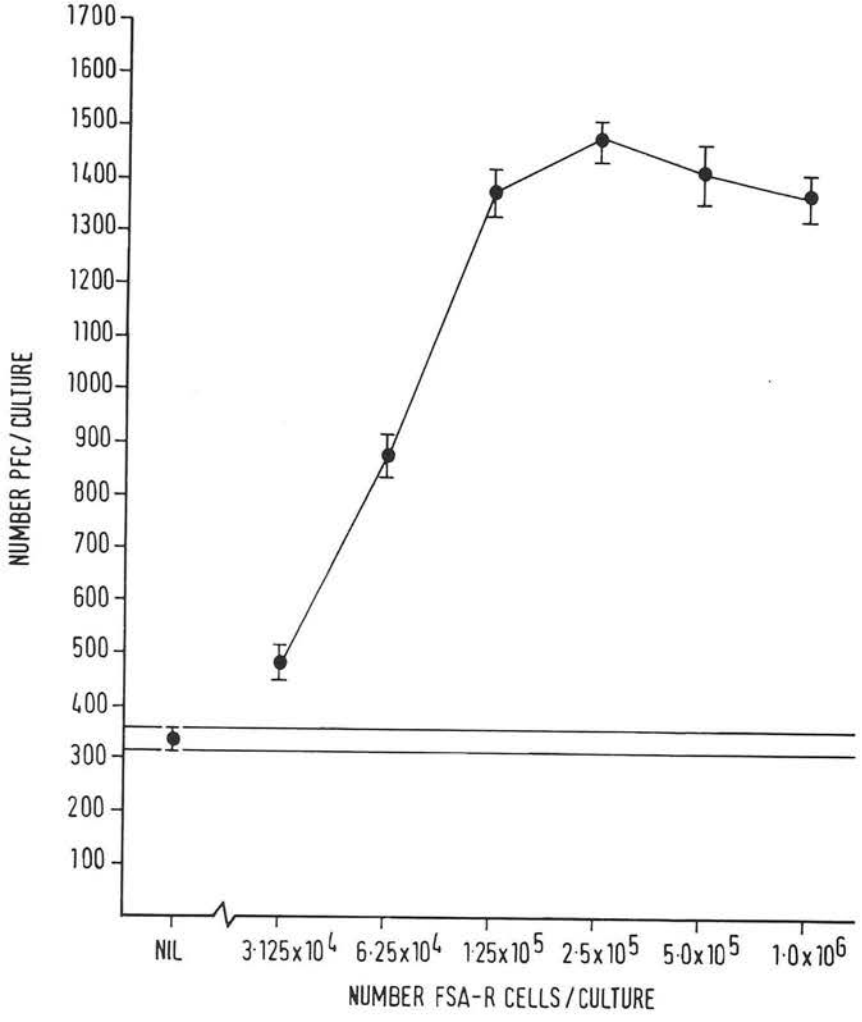

Figure 15 Effect of tumour-associated macrophages on the primary anti-CRBC PFC response of whole normal spleen cells. 5×10^6 whole normal spleen cells were incubated with various numbers of tumour-derived adherent cells and 10^7 CRBC, and the anti-CRBC PFC response generated was assayed on day 4. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 30 anti-CRBC PFC/culture.

3.0 CHARACTERIZATION OF THE TUMOUR-ASSOCIATED MACROPHAGE POPULATION RESPONSIBLE FOR ENHANCEMENT

3.1 Effect of anti-Ia serum plus complement treatment on the enhancing activity of tumour-associated macrophages

Monolayers of adherent tumour-associated macrophages were depleted of cells expressing Ia antigens by treatment with anti-Ia serum plus complement (Table XII). The results presented in Figure 16, show clearly that this treatment did not significantly alter the capacity of tumour-associated macrophages to enhance the primary anti-CRBC PFC response of whole normal spleen cells. Since anti-Ia serum plus complement treatment resulted in approximately 40% of cells being lost from the monolayers (Table XII), it is clear that if both Ia-negative and Ia-positive cells had possessed enhancing activity then such treatment would have shifted the dose response curve to the right. Since no such change was found it can be concluded that only Ia-negative cells are involved in enhancement.

3.2 Comparison of the enhancing activity of macrophages obtained from tumours grown in nu/+ or nu/nu mice

FSA-R tumours grown in either nu/+ or nu/nu animals both contained approximately 24% macrophages (Table IX). However, while macrophages obtained from tumours grown in nu/+ animals were approximately 50% I-A^k-positive, those obtained from tumours grown in nu/nu animals were less than 1% I-A^k-positive (Table IX).

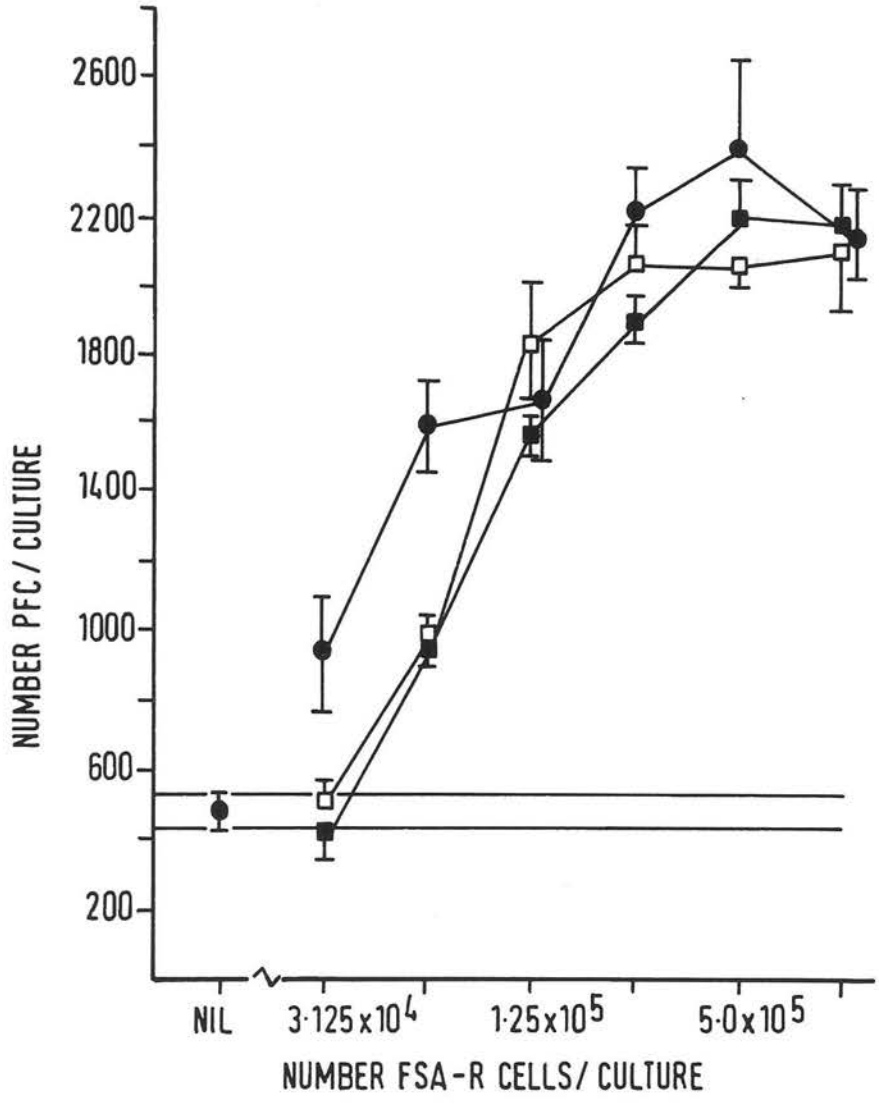

Figure 16 Effect of anti-Ia serum plus complement treatment on the enhancing activity of tumour-associated macrophages. 5×10^6 whole normal spleen cells were incubated with 10^7 CRBC and the anti-CRBC PFC response generated was assayed on day 4 (●). The effect of addition of various numbers of untreated (●), complement treated (□) or anti-Ia serum plus complement treated (■) tumour-derived adherent cells on the magnitude of the response generated was determined. Each point represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 70 anti-CRBC PFC/culture.

Nevertheless, macrophages obtained from tumours grown in nu/nu animals were as efficient as those obtained from tumours grown in nu/+ animals at enhancing the primary anti-CRBC PFC response of whole normal spleen cells (Figure 17). These results support the view that Ia-positive cells are not required for enhancement by tumour-associated macrophages.

3.3 Comparison of the enhancing activity of populations of tumour-associated cells separated by centrifugal elutriation

The cells present within enzymatically disaggregated tumour cell suspensions were found to be very heterogeneous with respect to cell size (Figure 18). Centrifugal elutriation was used to separate tumour cell suspensions into 5 fractions of differing modal diameter (Figure 19; Table XIII). More than 96% of the cells initially loaded were recovered following this procedure. The cells present in Fractions II-V were examined for their ability to enhance the anti-CRBC PFC response of whole normal spleen cells. The results presented in Figure 20 show that significant enhancing activity was found only in fractions II and III, the fractions which contained the majority of tumour-associated macrophages (Table XIII). Fractions IV and V which were composed mainly of tumour cells had no enhancing activity (Figure 20). It is interesting to note that although Fractions III contained only 13.4% Fc receptor-positive cells, it produced a similar degree of enhancement as Fraction II which contained 83.0% Fc receptor-positive cells (Table XIII; Figure 20). The

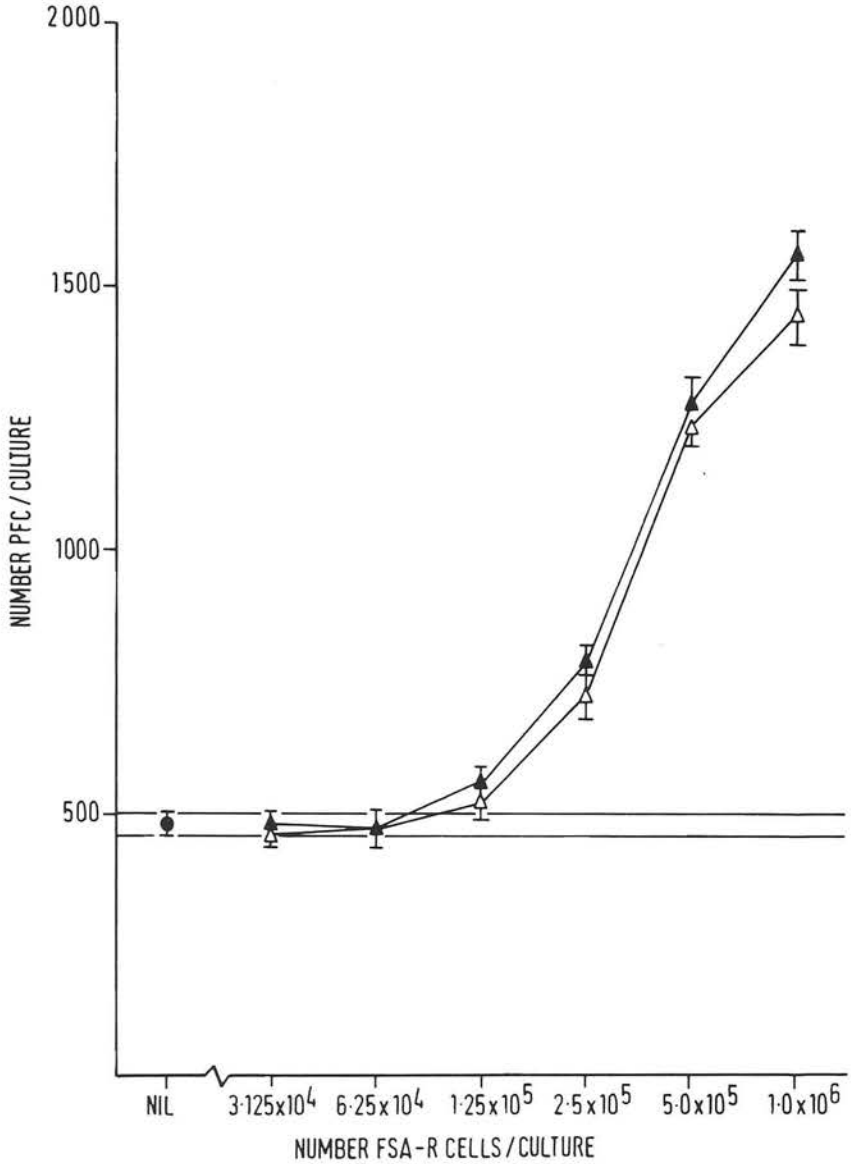

Figure 17 Comparison of the enhancing activity of macrophages obtained from tumours grown in nu/+ or nu/nu mice. 5×10^6 whole normal spleen cells were incubated with 10^7 CRBC and the anti-CRBC PFC response generated was assayed on day 4 (●). The effect of addition of various numbers of adherent cells derived from tumours grown nu/+ (▲) or nu/nu (△) mice on the magnitude of the response generated was determined. Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 60 anit-CRBC PFC/culture.

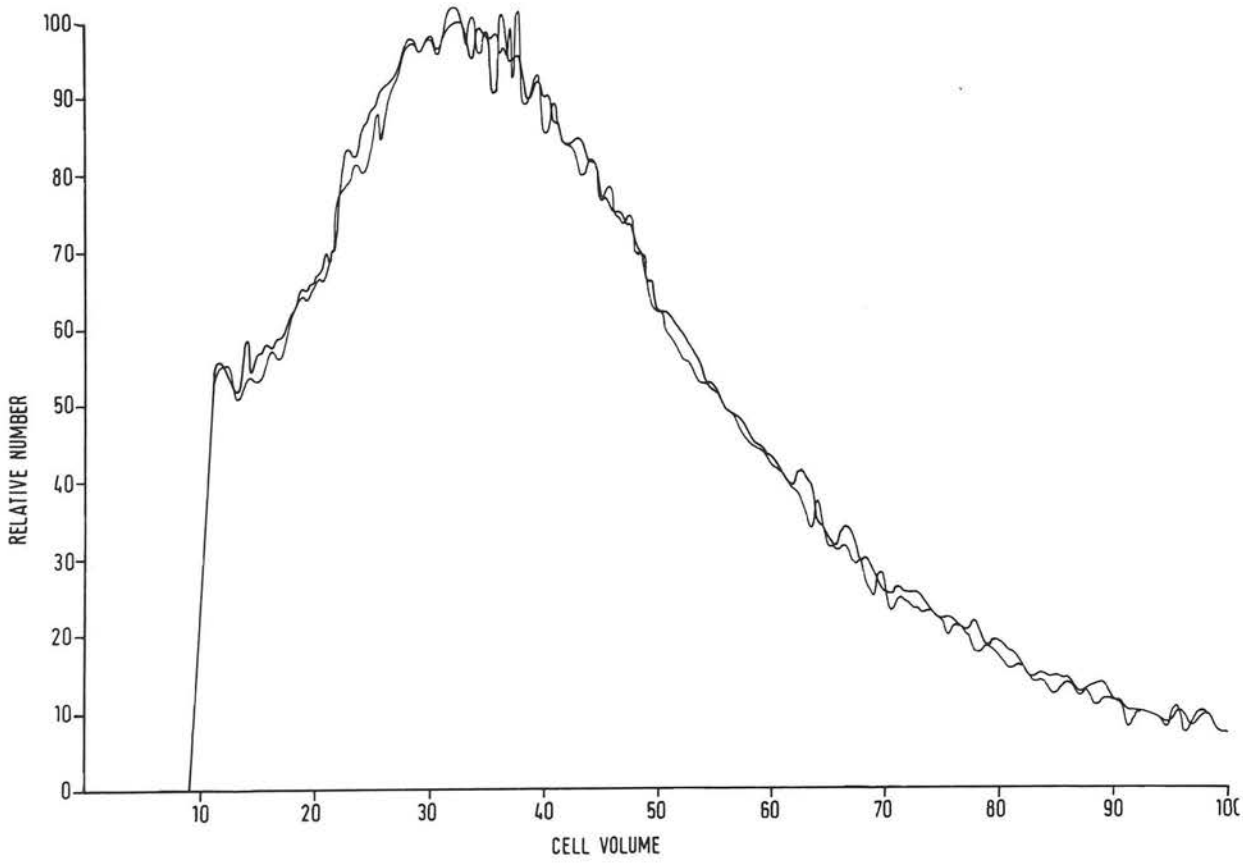

Figure 18 Coulter Channelyzer size distribution graph of enzymatically disaggregated FSA-R. Each X-axis scale division corresponds to 13.98 μm^3.

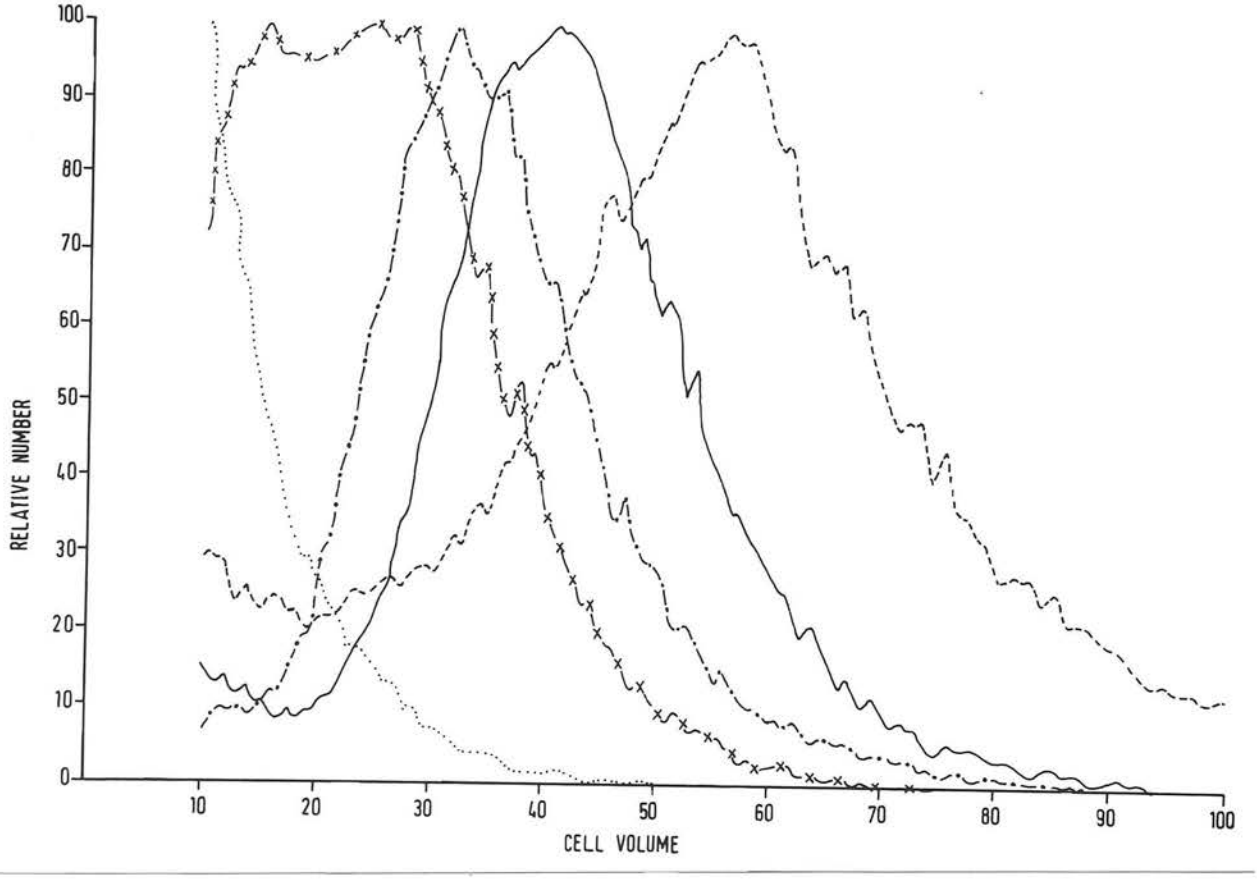

Figure 19 Coulter Channelyser size distribution graph of populations of tumour-associated cells separated by centrifugal elutriation. Fraction I - flow rate = 10.02 ml/min (·····), Fraction II - flow rate = 15.12 ml/min (-x-x-), Fraction III - flow rate = 22.97 ml/min (—·—·—), Fraction IV - flow rate = 28.46 ml/min (——), Fraction V - flow rate = 54.98 ml/min (— — —). Each X-axis scale division corresponds to 13.98 μm^3.

TABLE XIII Characterization of populations of tumour-associated cells separated by centrifugal elutriation.

	Flow rate (ml/min)	% total recovered cells	Modal cell diameter (um)[a]	% Fc receptor positive cells[b]	Morphological characterization[c]
Unfractionated	-	-	9.7	23.5	-
Fraction I	10.02	16.1	6.6	1.0	non-viable cells, cell debris erythrocytes, lymphocytes
Fraction II	15.12	20.3	8.6	83.0	Small macrophages lymphocytes tumour cells
Fraction III	22.97	29.6	9.6	13.4	Large macrophages tumour cells
Fraction IV	28.46	16.7	10.2	2.6	tumour cells
Fraction V	54.98	17.3	11.4	1.0	tumour cells

a Determined from Coulter Channelyser size distribution graph (Figure 19).

b Fc receptor-positive cells were identified by EA rosette formation.

c Determined from Giemsa stained cytocentrifuge preparations.

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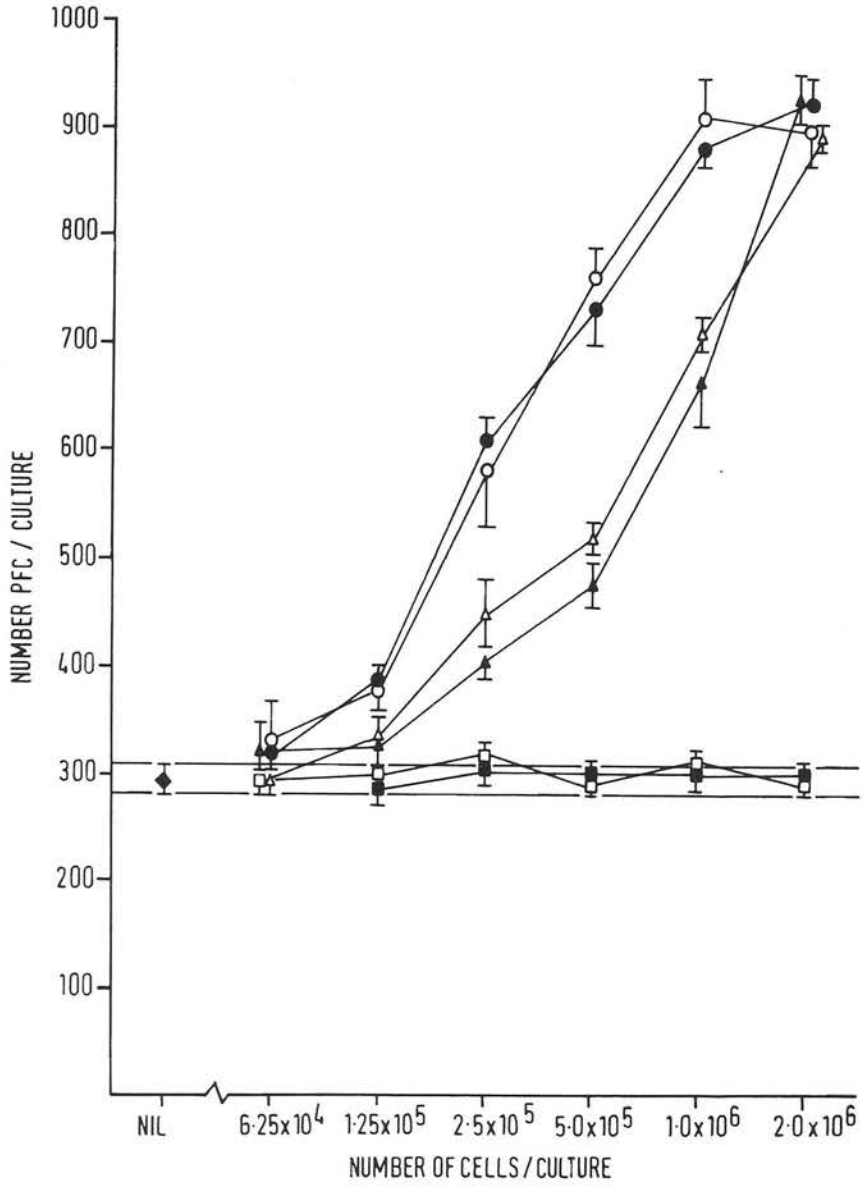

Figure 20 Enhancement of the primary anti-CRBC PFC response of whole normal spleen cells by populations of tumour-associated cells separated by centrifugal elutriation. 5×10^6 whole normal spleen cells were incubated with 10^7 CRBC and the anti-CRBC PFC response generated was assayed on day 4 (◆). The effect of the addition of various numbers of tumour-associated cells separated by centrifugal elutriation was determined. Whole unfractionated (●), adherent unfractionated (○), Fraction II (△), Fraction III (▲), Fraction IV (□), Fraction V (■). Each point represents the mean $\pm$ 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC always generated less than 40 anti-CRBC PFC/culture.

most likely interpretation of this finding is that enhancing activity is associated mainly with large macrophages which presumably predominate in fraction III but are fewer in fraction II. The finding that tumour-derived adherent cells were as efficient as the whole tumour cell suspension at enhancing the PFC response (Figure 20) argues against the possibility that the small tumour cells present in fractions II and III were responsible for enhancement.

4.0 ROLE OF SOLUBLE FACTORS IN MACROPHAGE-MEDIATED ENHANCEMENT

Macrophages are known to secrete a number of soluble factors (monokines) which may exert diverse effects on the immune response (reviewed by Unanue, 1981). The possibility that enhancement of the primary anti-CRBC PFC response of whole normal spleen by tumour-associated macrophages was mediated by soluble factors was investigated. Culture supernatants conditioned by tumour-derived adherent cells were found to be highly efficient at enhancing the PFC response (Figure 21). The magnitude of the enhancement obtained depended on the number of adherent cells used to condition the medium and whether supernatants were harvested after 24 or 48 hours. Culture medium conditioned for 48 hours was less efficient at enhancing the response than medium conditioned for 24 hours.

These results suggest that enhancement of the primary anti-CRBC PFC response of whole normal spleen cells by tumour-associated macrophages was mediated by soluble factors.

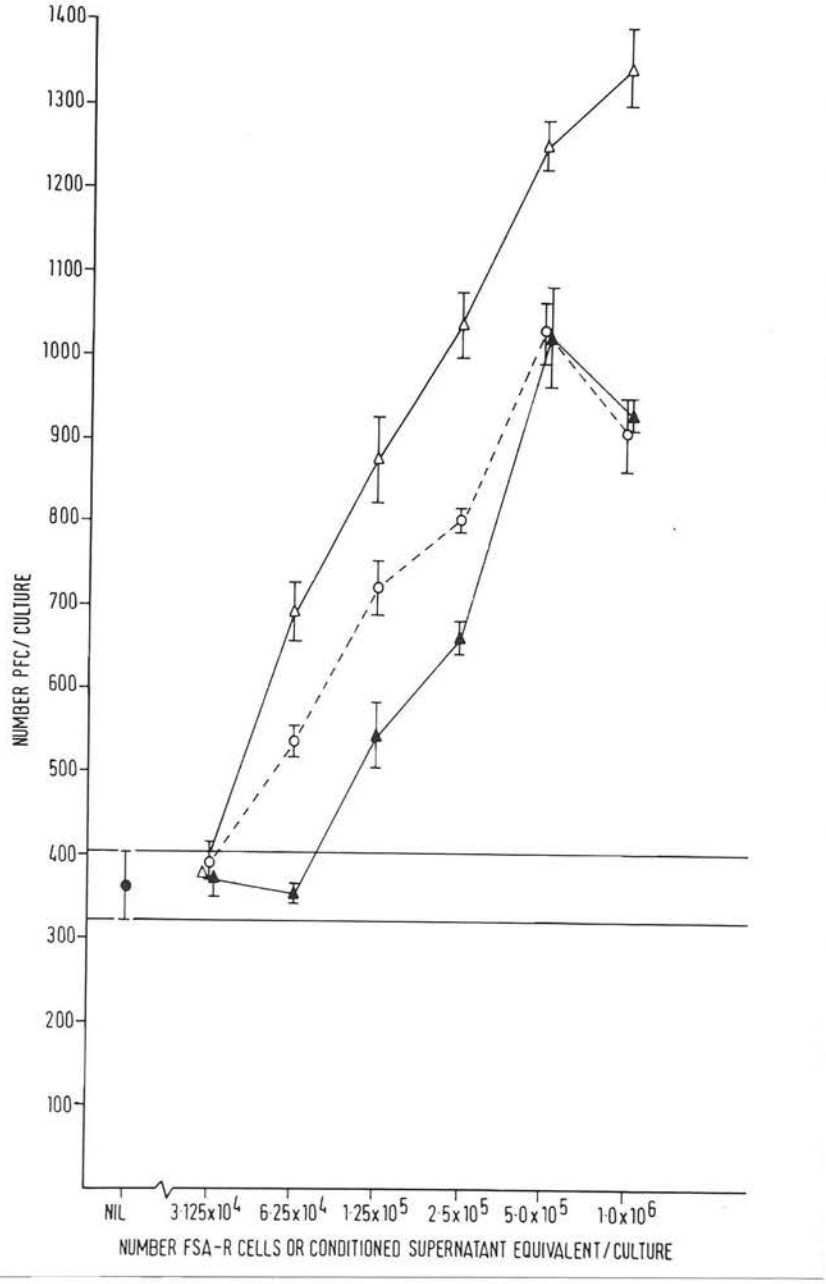

Figure 21 Enhancement of the primary anti-CRBC PFC response of whole normal spleen cells by macrophage-conditioned medium. 5×10^6 whole normal spleen cells were incubated with 10^7 CRBC and the anti-CRBC PFC response generated was assayed on day 4 (●). The effect of the addition of various numbers of tumour-derived adherent cells (○) or 50% (v/v) of medium conditioned by an equivalent number of tumour-derived adherent cells for 24 hours (△) or 48 hours (▲), was determined. Each point represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 80 anti-CRBC PFC/culture.

5.0 MACROPHAGE-MEDIATED ENHANCEMENT OF THE ANTI-CRBC PFC RESPONSE OF WHOLE NORMAL SPLEEN CELLS IN THE PRESENCE OF EXCESS T_H CELLS

5×10^6 whole normal spleen cells and 10^7 CRBC were cultured with or without 1×10^6 nylon-wool passed CRBC-primed spleen cells and the effect of the addition of tumour-derived adherent cells on the anti-CRBC PFC response generated was determined. The results presented in Figure 22 show clearly that while tumour-derived adherent cells enhanced the PFC response of cultures which did not receive added T_H cells, they had no significant effect on the response of those cultures which did receive added T_H cells.

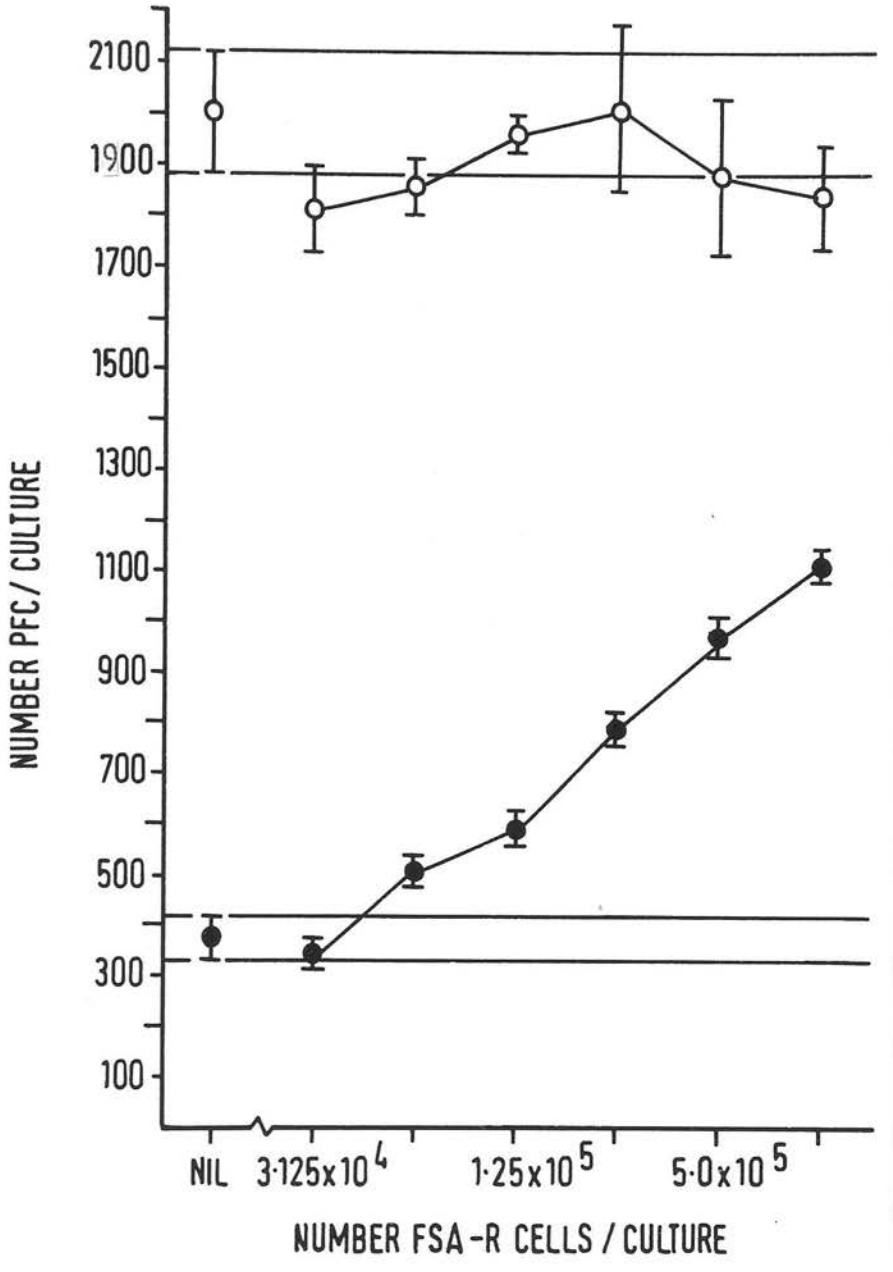

Figure 22 Macrophage-mediated enhancement of the anti-CRBC PFC response of whole normal spleen cells in the presence of excess T_H cells. 5×10^6 whole normal spleen cells and 10^7 CRBC were incubated with (○) or without (●) 1×10^6 nylon wool-passed CRBC-primed spleen cells and the anti-CRBC PFC response generated was assayed on day 4. The effect of the addition of various numbers of tumour-derived adherent cells to these cultures was determined. Each result represents the mean ± 1 SEM of triplicate cultures. Equivalent cultures which did not receive CRBC generated less than 50 anti-CRBC PFC/culture.

CHAPTER V - DISCUSSION

The preparation from solid tumour tissue of suspensions of viable single cells with minimum contamination by erythrocytes, aggregates, cell debris and other unwanted material is necessary in order that tumour-associated host cells can be accurately quantitated and their functional characteristics examined. There is however no universal method which is suitable for the disaggregation of all tumours and the advantages and disadvantages of any given method need to be carefully assessed in each experimental system. Ideally, the disaggregation procedure used should not enrich or deplete particular cell types, nor should it adversely affect the expression of markers used for the differential characterization of the diverse cellular components which make up the tumour. In addition, studies concerned with the functional characteristics of tumour-associated host cells must take into account the effect of tumour disaggregation and cell separation procedures on the parameter under investigation.

In the present study, an enzymatic disaggregation procedure employing a combination of Dispase and DNase was used to prepare single cell suspensions from tumour tissue. This procedure proved highly effective and resulted in almost complete disaggregation of tumour material. There is however no satisfactory method available for estimating the yield of cells recovered after such treatment and the composition of the tumour is represented only by those cells that survive the disaggregation procedure. It is quite possible that some cells may be preferentially lost, while others may not be easily released from stromal

constituents. However, cell suspensions may be considered representative if individual cellular components are present in the same proportions as in the intact tumour (Moore and Moore, 1977a). An excellent correlation was found between the percentage of Fc receptor-positive cells present in tumour sections as determined by EA absorption (Section I, 2.1) and the percentage of Fc receptor-positive cells identified in enzymatically disaggregated tumour cell suspensions by EA rosetting (Table I). It is therefore assumed that the ratio of the various cell types present in enzymatically disaggregated tumour cell suspensions was representative of that found within the intact tumour.

Proteolytic enzymes have been shown to alter both the expression of cell surface structures and dependent functional characteristics (Askenase and Hayden, 1974; Unkeless, 1977; Stahl et al., 1980). There was however no evidence that the enzymatic disaggregation procedure used in the present study had any deleterious effect on the properties of host cells necessary for their identification or characterization. Unfortunately it was not possible to compare the characteristics of enzymatically disaggregated tumour cells with equivalent cells obtained by some other procedure. Mechanical disruption of tumour tissue always gave preparations of low yield and poor viability. Populations of cells obtained under these conditions are likely to represent only those cells which were easily released from the tumour matrix and which were most resistant to the harsh dis-

aggregation procedure. However, the enzyme treatment used to disaggregate tumour tissue and separate out tumour-associated macrophages did not significantly alter the percentage of whole resident peritoneal cells which expressed Mac-1 (Table IV), or the percentage of adherent resident peritoneal cells which expressed Fc receptors (Table VI), Mac-1 (Table IV) or $I\text{-}A^k$ (Table VI). It is assumed therefore that the enzymatic disaggregation procedure did not alter the expression of these same characteristics on tumour-associated host cells.

The percentage of macrophages present in tumour cell suspensions has been estimated by morphology (Talmadge et al., 1981), histochemical staining (Svennevig and Saar, 1979), Fc and C3 receptor expression (Evans, 1972; Kerbel et al., 1975; Moore and Moore, 1977a; Wood and Morantz, 1979; Moore and McBride, 1980) and various functional criteria including adherence (Evans, 1972; Mantovani, 1981) phagocytosis (Evans, 1972; Moore and Moore, 1977a; McBride et al., 1982) and colony formation in vitro (Nash et al., 1981). It is unlikely however that any one method will identify all macrophages in a mixed population of cells while excluding from the determination all non-macrophage cell types. The macrophage phenotype is very heterogeneous and likely to depend on differentiation stage and previous exposure to modulating signals (Dougherty and McBride, 1984). Moreoever, many of the markers used to identify macrophages are by no means unique to this cell type. For

example, one of the more notable features of Fc receptors is their ubiquity. In addition to macrophages, they have also been detected on B lymphocytes, NK cells, K cells,eosinophils, neutrophils, basophils and some T lymphocytes (Kerbel and Davies, 1974; Basten et al., 1975). Several non-immune cell types may also express Fc receptors including liver parenchymal cells (Hopf et al., 1976), certain gut epithelial cells (Guyer et al., 1976) and some malignant cells (Noltenius, 1981; Svennevig and Andersson,1982). There is also the possibility that "released" Fc receptors may be passively absorbed by Fc receptor-negative cells. Such an occurrence has apparently been demonstrated for thymocytes (Rubin and Hertel-Wulff, 1975). Similarly, although macrophages are particularly efficient at phagocytosis many other diverse cell types may express this function. For example, latex particles are rapidly taken up by neutrophils and have been used extensively to study endocytosis by L-cells (Rabinovitch, 1969). However, with cell suspensions derived from the FSA-R tumour it is likely that the vast majority of cells expressing Fc receptors or phagocytosing latex particles are indeed macrophages. Lymphocyte infiltration of the tumour was found to be very modest, with average values only around 5% (Moore and McBride, 1983). Similarly, neutrophils were only rarely observed in cell suspensions prepared from non-nec rotic tumour tissue (Moore and McBride, 1983). In addition, neoplastic cells isolated by centrifugal elutriation did not express Fc receptors (Table XIII).

On the basis of Fc receptor expression and latex phagocytosis 20-25% of enzymatically disaggregated tumour cells may be classified as macrophages (Table I). The percentage of phagocytic cells was, on average, 4.5% less than the percentage of Fc receptor-positive (Table I) although almost all cells identified by either of these markers had typical macrophage morphology. The reason for the difference in the percentage of macrophages identified by these two different techniques is not clear. However, phagocytic cells were observed to differ greatly in the number of latex particles they ingested and it is possible that the conditions employed in the latex phagocytosis assay did not allow for the detection of the more poorly phagocytic cells.

Macrophages were enriched from tumour cell suspensions by virtue of their capacity to adhere tightly to glass or plastic surfaces in the presence of 0.1% Dispase and 20% FCS (Moore and McBride, 1980). The presence of proteolytic enzymes enhances macrophage spreading and effectively prevents tumour cells from adhering (Evans, 1973). Dispase is particularly useful in this respect since its activity is not abolished by serum proteins (Moore and McBride, 1980). The presence of serum proteins such as fibronectin promotes macrophage adherence while inhibiting the adherence of lymphocytes and other cells (Rabinovitch and DeStefano, 1973; Mosier, 1981). Under these conditions approximately 17.6% of cells present in the tumour cell suspension were adherent (Figure 2). More than 90% of these could be

classified as macrophages on the basis of Fc receptor expression and latex phagocytosis (Table I). The small percentage of non-macrophage cells present among the adherent population appeared by morphological criteria to be lymphocytes or tumour cells which were usually bound tightly to adherent macrophages.

The accumulation of macrophages within the FSA-R tumour appears to be a well regulated process. The percentage of tumour-associated cells which expressed Fc receptors remained constant throughout the observed period of tumour growth (Table III), and during serial passage from one syngeneic host to another (Table II). The mechanisms responsible for determining and maintaining the characteristic macrophage content of the tumour are unknown. However, it seems unlikely that mediators of acute inflammation derived from necrotic tissue are involved since large necrotic tumours contained the same percentage of Fc receptor-positive cells as smaller non-necrotic tumours (Table III). In addition, the finding that tumours grown in nu/nu mice contained the same percentage of macrophages as tumours grown in nu/+ littermate controls (Table IX) suggests that T cells or their products are also not involved in macrophage accumulation.

Monoclonal antibodies constitute a powerful tool for the analysis of cell surface antigen expression and have proven extremely useful in the identification and separation of various

functionally distinct lymphocyte subsets (Ledbetter and Herzenberg, 1979). A similar application of hybridoma technology to macrophages could provide a means to identify and separate these cells from complex cell mixtures. In recent years a substantial number of anti-macrophage monoclonals have been developed and these are already proving invaluable reagents for the study of macrophage cell surface antigen structure, differentiation and function (reviewed by Dougherty and McBride, 1984).

The rat antibody M1/70 was the first monoclonal to identify an antigen expressed by myeloid cells but not lymphocytes (Springer et al., 1979). The antigen defined by M1/70 has been designated Mac-1 (Springer et al., 1979) and more recently, several other monoclonal antibodies to it have been obtained (Mellman et al., 1980; Springer et al., 1982; Sanchez-Madrid et al., 1983). Although raised against murine macrophages, M1/70 crossreacts with human Mac-1, an antigen which shows an identical distribution to murine Mac-1 (Ault and Springer, 1981), and which is apparently equivalent to the OKM1 and Mo1 antigens (Beller et al., 1982).

The Mac-1 antigen is composed of two subunits, an α subunit with a molecular weight of 170,000 daltons and a β subunit of 95,000 daltons. The α and β subunits are tightly, but non-covalently, linked in an α_1, β_1 complex (Kurzinger and Springer, 1982; Kurzinger et al., 1982). Monoclonal

antibodies against the Mac-1 antigen bind to the α subunit (Sanchez-Madrid et al., 1983). The LFA-1 antigen on lymphocytes has a different α subunit associated with the same β subunit (Kurzinger et al., 1982; Sanchez-Madrid et al., 1983) and thus antibodies to the β subunit have a broader pattern of cellular reactivity (Sanchez-Madrid et al., 1983). Both the α and β subunits are glycosylated and have surface exposure (Kurzinger and Springer, 1982).

It has been proposed that Mac-1 constitutes a universal macrophage marker (Ho and Springer, 1982). The antigen is expressed on more than 95% of resident or elicited peritoneal macrophages (Ho and Springer, 1982). It is also present on splenic macrophages in both the red pulp and marginal zones (Ho and Springer, 1982), and on lymph node and medullary cord macrophages (Flotte et al., 1983). Histocytes in the lamina propria of the intestine and alveolar macrophages are positive, but Kupffer cells are negative (Flotte et al., 1983). Eight independent macrophage cell lines ranging in phenotype from mature (J774 and $P388D_1$) to relatively immature (WEHI-3) all expressed Mac-1 (Ralph et al., 1983).

Mac-1 appears to be acquired by macrophages at an early stage in their differentiation from immature precursor cells. Studies in humans have shown that GM-CFC are Mac-1-negative or express only low amounts of Mac-1 (Griffin et al., 1982).

Similarly, the immature myelomonocytic leukemia cell line M1 is Mac-1-negative (Ralph,et al.,1983). However treatment of M1 with phorbol ester or lymphokines caused these cells to differentiate and acquire macrophage characteristics including the Mac-1 antigen (Ralph et al., 1983).

Mac-1 expression is however not entirely unique to macrophages and the antigen is also found in appreciable amounts on exudate granulocytes and committed granulocyte precursors in the bone marrow (Springer et al., 1979), and on cells with natural killer activity obtained from the nylon wool non-adherent fraction of peritoneal exudates (Holmberg et al., 1981) or isolated directly from human peripheral blood (Ortaldo et al., 1981; Zarling et al., 1981; Ault and Springer, 1981). Alloactivated or cloned NK cells may be either Mac-1/OKM1 positive (Krensky et al., 1982; or negative (Brooks et al., 1982; Hercend et al., 1983; Sheehy et al., 1983).

Blocking studies suggest a close association or identity between Mac-1 and the type 3 complement receptor (CR_3) specific for C3bi. For example, the M1/70 anti-Mac-1 monoclonal antibody was found to strongly inhibit rosette formation between macrophages and complement coated erythrocytes (Beller et al., 1982). Lack of inhibition by a panel of eight other antibodies demonstrated the specificity of blockade. Macrophages bear receptors for both C3b (CR_1) and C3bi (CR_3) (Ross, 1980). However, when erythrocytes

bearing only C3b or C3bi were prepared with homogeneous complement components, it was found that M1/70 inhibited CR_3 but not CR_1 mediated binding (Beller et al., 1982). Similar results were obtained with human granulocytes (Beller et al., 1982). The most likely interpretation of these findings is that Mac-1 is the CR_3. In this connection it is interesting to note that Mac-1 and the CR_3 appear to have an identical cellular and tissue distribution (Ross, 1980; Fearon and Wong, 1983).

In the present study, an attempt was made to use Mac-1 expression as a marker to identify and quantitate tumour-associated macrophages. In control experiments, an excellent correlation was found between the percentage of whole or adherent resident peritoneal cells which expressed Fc receptors and/or phagocytosed latex particles, and the percentage of Mac-1-positive cells identified using an indirect immunoperoxidase assay (Table I). Similar results were obtained for whole or adherent C.parvum -elicited peritoneal cells (Table I). This was as expected since all of these techniques should identify mainly macrophages. In contrast, although approximately 24.5% of enzymatically disaggregated tumour cells expressed Fc receptors and around 20% phagocytosed latex particles, less than 1% of these cells expressed Mac-1 (Table I). Similarly, more than 90% of tumour-derived adherent cells expressed Fc receptors or phagocytosed latex, but only around 4% expressed Mac-1 (Table I).

The possibility was considered that the enzyme treatment

used to disaggregate tumour tissue may have had a deleterious effect on Mac-1 expression by tumour-associated macrophages. However, tumour-derived adherent cells cultured for up to 18 hours did not re-express Mac-1 (Table V). In addition, Mac-1 expression by whole or adherent resident peritoneal cells was unaltered by enzyme treatment (Table IV). This finding is in agreement with recent studies by Unkeless and Springer (1984) which demonstrated that treatment of macrophages with trypsin or papain did not alter Mac-1 expression. Taken together these results suggest that it is not possible to attribute the very low level of Mac-1 expression on tumour-associated macrophages to the effect of the enzymatic disaggregation procedure.

The lack of Mac-1 expression by tumour-associated macrophages suggests that these cells do not express CR_3. In this connection it is interesting that neither B lymphocytes or macrophages harvested from acutely rejecting cardiac allografts in rats formed a significant number of rosettes with complement coated erythrocytes (Tilney et al., 1975). In addition, Moore and Moore, (1977a)in a study of the nature of the host-derived cells present in five different transplanted rat tumours, found that very few cells formed rosetts with complement-coated erythrocytes despite the presence of significant numbers of macrophages. Finally, Sher et al. (1980) found that relatively few of the Ia-positive macrophages present in peritoneal exudates induced by either live Listeria monocytogenes or a lymphokine elaborated by immune T cells

expressed complement receptors.

On the basis of the available data any explanation for the apparent absence of Mac-1 on FSA-R tumour-associated macrophages can be no more than speculation. It is however interesting to note that the CR_3 receptor has been implicated in the phagocytosis of immune complexes (Ross, 1980). It is quite likely that immune complexes could occur within highly immunogenic tumours such as FSA-R. These immune complexes could perhaps bind to and block the CR_3. Alternatively, although very little information is available on the synthesis and turnover of complement receptors (Fearon, 1984), it is possible that interaction of CR_3 with immune complexes could lead to internalization of the receptor reducing its surface expression to below detectable levels. In this connection, it would be most interesting to compare Mac-1 expression on macrophages obtained from tumours of differing immunogenicities. An alternative explanation for the absence of Mac-1 on tumour-associated macrophages could be that these cells represent a distinct subpopulation of macrophages which do not express CR_3. The finding that Kupffer cells do not express Mac-1 (Flotte et al., 1983) lends some support to this possibility.

When the function of surface markers is known, their presence or absence on a particular cell type takes on a greater significance. CR_3 (Mac-1) enables macrophages to bind cells or

particles opsonized with complement component C3bi (Fearon, 1984). Moreover, on activated macrophages, the CR_3 mediates phagocytosis (Michl et al., 1979) and on resident macrophages, the CR_3 is synergistic with the Fc receptor for phagocytosis (Bianco and Nussenzweig, 1977). Similarly, when target cells are coated with C3bi in addition to IgG lysis by ADCC effectors is greatly enhanced (Perlmann et al., 1981). From these results it is clear that the absence of CR_3 from the surface of tumour-associated macrophages may have important implications with respect to the anti-tumour activity of these cells in situ.

Macrophages can be grouped as to whether they express or do not express detectable Ia antigens (reviewed by Unanue, 1981). The expression of $I\text{-}A^k$ by tumour-associated macrophages was examined using an indirect immunoperoxidase technique. Approximately 10% of enzymatically disaggregated tumour cells and around 50% of tumour-derived adherent cells were found to express the $I\text{-}A^k$ antigen (Table I). By morphological criteria most of these $I\text{-}A^k$ positive cells appeared to be typical macrophages, although such cells did show considerable heterogeneity both with respect to their expression of various morphological characteristics (size, nuclear shape, cytoplasmic structures etc) and the density of immunoperoxidase staining, presumably reflecting differences in the amount of $I\text{-}A^k$ expressed per cell (Plate I). Assuming that approximately 20% of enzymatically disaggregated tumour cells are macrophages (see above) and that all $I\text{-}A^k$-

positive cells present in the tumour cell suspension are also macrophages, then one would expect approximately 50% of tumour-associated macrophages to express I-A^k. Since the tumour-associated macrophages enriched from tumour cell suspensions by adherence were indeed approximately 50% I-A^k-positive (Table I), it would seem that the separation procedure used to isolate these cells does not specifically enrich or deplete I-A^k-positive cells. It is likely therefore that the population of adherent macrophages is representative of those found within the enzymatically disaggregated tumour cell suspension.

The percentage of macrophages which express Ia determinants varies from tissue to tissue, although in the absence of external stimuli, the ratio for a given tissue is remarkably constant (reviewed by Unanue, 1981). In order to maintain this basal level of Ia expression there is a need for a continuous influx of young macrophages into the tissue (Scher et al., 1982). It seems that Ia expression develops mainly in young macrophages and unlike the expression of class I molecules, it is a transient event which is rapidly lost with time (Beller and Unanue, 1981 ; Scher et al., 1982).

The percentage of tumour-associated macrophages which expressed I-A^k remained constant during both the observed period of tumour growth (Table VIII) and serial passage from one syngeneic host to another (Table VII). As with other macrophages, the expression of I-A^k by the tumour-associated population appears

to be a transient event. The percentage of such cells expressing I-A^k decreased rapidly with time in culture and after 3 days only around 6% of the macrophages continued to express the antigen (Figure 3). No further reduction in I-A^k expression was found if the cells were cultured for 4 days. Those cells which continued to express I-A^k after 4 days in culture appeared by morphological criteria to be typical macrophages. Whether they constitutively express I-A^k or represent a subpopulation which loses I-A^k more slowly is unknown.

Since I-A^k expression by tumour-associated macrophages appears to be a transient event, the question arises as to what mechanisms operate *in situ* to induce and maintain the antigen on such a large percentage of these cells. At least three distinct mechanisms have been implicated in the regulation of macrophage Ia expression:

1) some agents that trigger phagocytosis can slow down or prevent the loss of Ia (Beller and Unanue, 1981);

2) T cell-derived lymphokines can induce Ia-negative macrophages to express Ia (Scher *et al.*, 1980, 1982);

3) macrophage Ia expression can be actively down regulated by various inhibitory molecules including prostaglandins (Snyder *et al.*, 1982).

The possible involvement of T cells in the regulation of

I-A^k expression by tumour-associated macrophages was investigated by comparing the expression of I-A^k by macrophages obtained from tumours grown in nu/+ or nu/nu mice. It was found that although tumours grown in nu/+ or nu/nu animals both contained the same percentage of macrophages (Table IX), very few of the macrophages present in tumours grown in nu/nu animals expressed detectable I-A^k (Table IX). These findings suggest that although T cells or their products are probably not involved in determining the macrophage content of the tumour, they do nevertheless play an important role in regulating I-A^k expression by these cells.

In most experimental systems, the generation of an effective immune response has been shown to require cooperation between immunocompetent lymphocytes and non-lymphoid accessory cells (reviewed by Unanue, 1972, 1981). Although our understanding of this interaction is far from complete, it seems that T_H cells are not activated as the result of a simple encounter with native antigen (Ishizaka and Adachi, 1976; Pierres and Germain, 1978). Instead, accessory cells are required to take up and "process" antigenic material and "present" the appropriate epitopes to T cells in association with Ia determinants (reviewed by Unanue et al., 1984). A large number of studies have demonstrated that Ia-positive macrophages, normally obtained from the spleen or peritoneal cavity, can perform this accessory cell function (reviewed by Unanue, 1981).

Ia-positive putative accessory cells have been identified within the inflammatory lesions associated with a number of pathological conditions including inflammatory bowel disease (Selby et al., 1981, 1983); infectious diseases such as schistomiasis (Stadecker et al., 1982); and several diseases of unknown aetiology including rheumatoid arthritis (Klareskog et al., 1981; Winchester and Burmester, 1981; Poulter et al., 1982) and sarcoidosis (Mishra et al., 1983). Of these various conditions, the synovial inflammation associated with rheumatoid arthritis has probably received most attention. Large numbers of HLA-DR-positive macrophages have been identified within the hyperplastic synovial lining (Poulter et al., 1982; Burmester et al., 1983) and interdigitating cells appear to accumulate in the deeper stroma (Poulter et al., 1982). Combination immunohistochemical analysis of the synovial lining has revealed a close association between these HLA-DR-positive cells and infiltrating T cells expressing the helper phenotype (Duke et al., 1982). Moreover, functional studies have shown that HLA-DR-positive non-T cells from the rheumatoid synovium can stimulate autologous mixed lymphocyte reactions (Forre et al., 1982). Such findings have lead to the suggestion that the pathogenesis of rheumatoid arthritis may in part be due to the generation of local immune responses (Janossy et al., 1981).

Although the presentation of antigen in association with Ia determinants on the surface of an accessory cell is essential

for the initiation of T cell responses, it is important to realise that the generation of an effective immune response requires the involvement of other diverse signals which promote T cell clonal expansion and differentiation (reviewed by Howie and McBride, 1982a). Thus in order to define a role for I-A^k-positive tumour-associated macrophages in the generation of anti-tumour immune responses, it is necessary to show that these cells possess functional accessory cell activity.

Many different experimental approaches have been exploited to examine macrophage accessory cell function (reviewed by Unanue, 1981). In vitro systems have proven particularly useful in this respect since the effect of the removal or manipulation of macrophages on the generation of an immune response can be studied using uniform well defined cell populations and standardized conditions. The antibody response to heterologous erythrocytes has been most frequently used probably because of its ease of production (Mishell and Dutton, 1966, 1967; Marbrook, 1967) and analysis (Jerne and Nordin, 1963; Cunningham, 1965; Cunningham and Szenberg, 1968; Jerne et al., 1974).

The requirement for an adherent accessory cell population as well as T cells and B cells for the generation of an efficient in vitro antibody response to erythrocyte antigens was first described by Mosier (1967) and has subsequently been confirmed by a large number of different groups (e.g. Pierce, 1969;

Roseman, 1969; Shortman et al., 1970; Feldmann and Palmer, 1971; Sjoberg et al., 1972; Niederhuber, 1978; Niederhuber and Allen, 1980; Wong and Hercowitz, 1979). More recently, a similar requirement for adherent accessory cells was demonstrated for in vitro antibody reponses to soluble protein antigens (Pierce et al., 1974), or TNP conjugated to synthetic peptides or proteins such as haemocyanin (Hodes and Singer, 1977; Hodes et al., 1978; Singer et al., 1978). Only very occasional studies have found no requirement for accessory cells in the generation of an in vitro antibody response (Pierce, 1969; Bluestein and Pierce, 1973a,b; Katz and Unanue, 1973) and in most instances such findings can be readily ascribed to insufficient depletion of accessory cells from the responding lymphoid population (Unanue, 1981).

In the present study, whole normal spleen cells were found to generate a primary anti-CRBC PFC response in vitro, the magnitude of which depended on both the antigen dose used and the period of time in culture (Table X). Maximum responses were consistently obtained when 5×10^6 whole normal spleen cells were cultured for 4 days with 10^7 CRBC.

If the responding spleen cells were depleted of accessory cells by passage through a Sephadex G-10 column (Ly and Mishell, 1974) (Table XI) prior to culture, then the magnitude of the anti-CRBC PFC response generated was considerably reduced

(Figure 4). However, merely reducing responsiveness by a depletion procedure known to remove accessory cells cannot be taken as definitive proof that these cells are required for the response. Clearly, Sephadex G-10 does not deplete only accessory cells. Indeed, only 35-40% of the cells initially applied to the column were recovered following this procedure. Although the recovered cell population had the same T cell to B cell ratio as the original spleen cell suspension (Table XI), it is possible that the separation procedure may have selectively depleted certain populations of T cells and B cells. In addition to macrophages, the passage of murine spleen cell suspensions through Sephadex G-10 has been reported to deplete antibody-forming cells (Ly and Mishell, 1974), some of the cells which mediate ADCC (Pollack et al., 1976) and activated T_S cell blasts (Pickel and Hoffman, 1977). Thus in order to establish a requirement for accessory cells in the generation of a primary anti-CRBC PFC response it was necessary to show that the Sephadex G-10 depleted response could be reconstituted by the addition of a source of accessory cells.

Adherent resident peritoneal cells have been shown in many studies to constitute a readily obtainable source of accessory cells (reviewed by Unanue, 1981). As indicated in Figure 4, the primary anti-CRBC PFC response of Sephadex G-10 passed normal spleen cells could be reconstituted in a dose-dependent fashion by the addition of adherent resident peritoneal cells. Maximum

reconstitution was obtained in cultures which contained approximately 2% adherent resident peritoneal cells. Cultures which received more than this number of cells generated somewhat lower responses.

From these studies it can be concluded that the generation of an anti-CRBC PFC response in vitro is absolutely dependent upon the presence of accessory cells. Consequently, the capacity of a population of cells to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells can be taken as a measure of accessory cell activity.

Adherent macrophages derived from the FSA-R tumour constituted an excellent source of accessory cells and were able to fully reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed normal spleen cells (Figure 5). Maximum reconstitution was once again obtained in cultures which received approximately 2% macrophages. However, it is clear that tumour-associated macrophages produced significant reconstitution over a narrower range of cell concentrations than resident peritoneal macrophages. The reasons for such differences are unclear but presumably relate to differences in the number of various distinct macrophage subsets within the two populations.

The accessory cells present within the tumour-associated

macrophage population were further characterized. It was found that treatment of tumour-associated macrophages with anti-Ia serum plus complement effectively depleted those cells bearing Ia determinants (Table XII) and the remaining mainly Ia-negative macrophages were unable to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells (Figure 6). The use of various macrophage doses rules out the possibility that this finding was the result of an inappropriate number of accessory cells remaining in the tissue culture wells after anti-Ia serum plus complement treatment. Instead, it appears that Ia-positive cells are required for the expression of accessory cell activity by tumour-associated macrophages. This conclusion is strongly supported by the finding that macrophages obtained from FSA-R tumours grown in nu/nu animals not only lacked detectable I-A^k antigen (Table IX) but were also unable to reconstitute the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells (Figure 7).

Although quite likely, it is not possible from these results to state definitively that the accessory cell function of FSA-R tumour-associated macrophages in the reconstitution of the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells involves presentation of antigen in association with Ia determinants. It is possible that Ia-positive macrophages perform some other function unrelated to antigen presentation. For example, several reports have demonstrated that

although the generation of a primary Tc cell response against tumour cells or allogeneic cells required the presence of Ia-positive macrophages, it did not seem to matter whether these were syngeneic or allogeneic to the responding lymphocytes (Yamashita and Hamaoka, 1979; Pettinelli et al., 1980). In order to formally demonstrate that tumour-associated macrophages present antigen it would be necessary to investigate the requirement for I-region identity between the accessory cell population and the responding lymphocytes using various congen ic mouse strains.

One of the most important factors limiting the number of PFC generated in cultures of whole normal spleen cells and antigen is the number of T_H cells which can recognise and respond to the antigen (Gisler et al., 1981). As indicated in Figure 8, the addition of nylon-wool passed CRBC-primed spleen cells to cultures of whole normal spleen and CRBC caused a dose-dependent increase in the number of anti-CRBC PFC generated. Maximum enhancement was found in cultures which received 5×10^5 nylon wool-passed CRBC-primed spleen cells, and no further increase in the response generated was found if more than 5×10^5 cells were added. Presumably at this point T_H cells are in excess and it is number of responding B lymphocytes or accessory cells which limit the magnitude of the response generated.

The question of how the "help" signal is delivered to the appropriate B cell is still the subject of some considerable debate. It is likely that more than one pathway exists and the particular pathway activated will probably depend on such factors as the dose and form of antigen, the degree of involvement of non-antigen specific lymphostimulatory mediators and the extent of priming (Howie and McBride, 1982a).

There is some evidence that interaction between T_H cells and B cells is a localized phenomenon involving direct cell contact (Phillips and Waldmann,1977). This was shown using a limiting dilution system in which very low numbers of Keyhole Limpet Haemocyanin (KLH)-specific T_H cells were added to cultures containing excess TNP-primed B cells. Some wells contained no T_H and failed to produce antibody to TNP when challenged with TNP-KLH. In those wells which did contain T_H the number of B cell clones generating antibody against TNP was determined on the basis of the isoelectric focusing pattern of the anti-TNP antibody produced. It was found that the observed distribution of anti-TNP B cell clones compared well with estimated numbers of T_H cells added per well implying that a single T_H cell can help only one B cell.

Antigen-specific T cell-derived soluble helper factors have been described in a limited number of systems. For instance, Feldmann and Basten (1972) demonstrated that the IgM response of

DNP-primed B cells to DNP-KLH was the same irrespective of whether KLH-specific T_H cells were mixed together with the B cells or if the two populations were separated by a cell impermeable membrane. The most likely interpretation of this finding is that T cells released a soluble helper factor which diffused into the B cell compartment. A similar T cell-derived factor with activity in vivo has also been described (Taussig, 1974). In this study, SRBC primed T cell which were challenged in vitro with antigen, produced a soluble mediator which could substitute for T cells in the response of unprimed B cells to SRBC in irradiated recipients.

Although they are antigen-specific, T cell-derived helper factors do not possess immunoglobulin constant region epitopes. They have however been described as having idiotypic determinants that are common to antibodies (Mozes and Haimovich, 1979) and do have Ia determinants which could be responsible for the MHC-restricted activity found with some of these factors (Shiozawa et al., 1977).

In contrast to the vast amount of data which has been collected on the role of macrophages in the induction of T_H cells (reviewed by Unanue, 1972, 1981), very little is known about the role of accessory cells in cooperation between T_H cells and B cells for the generation of an antibody response.

Where it has been studied accessory cells appear to be at least desirable if not obligatory for effective cooperation (Feldmann and Basten, 1972; Howie and Feldmann, 1978; Feldmann et al., 1978). In the present study, cooperation between CRBC-primed T cells and normal B cells in the generation of an anti-CRBC PFC response in vitro was found to be absolutely dependent upon the presence of accessory cells (Figure 9). If these cells were removed by passage through a Sephadex G-10 column the response was considerably diminished. However, this depleted response could be reconstituted by the addition of accessory cells, and tumour-associated macrophages could provide this accessory cell function (Figure 9). As with anti-CRBC PFC responses generated in the absence of added T_H (Figure 5), maximum reconstitution was obtained in cultures which contained approximately 2% macrophages.

Cooperation between carrier-primed T_H cells and normal hapten-specific B cells in the generation of an anti-hapten antibody response in vitro also required the presence of accessory cells. Whole normal spleen cells cultured with TNP-CRBC generated a rather small anti-TNP PFC response (Figure 10). However, this response was enhanced in a dose-dependent fashion by the addition of nylon wool-passed CRBC-primed spleen cells (Figure 10). These results indicate that carrier-primed T_H cells can cooperate with normal B cells to generate an anti-hapten antibody response in the presence of hapten-carrier

conjugates. If accessory cells were removed by passage of the responding spleen cells through a Sephadex G-10 column then the anti-TNP response generated was greatly decreased (Figures 11 and 12). However this depleted response could be reconstituted by the addition of either resident peritoneal macrophages (Figure 11) or tumour-associated macrophages (Figure 12).

One of the major problems of tumour immunology is to arrive at an understanding of the various cellular interactions that may occur between T and B lymphocytes and accessory cells in the generation of an anti-tumour immune response. Part of the difficulty with this aspect of tumour immunology arises from the problems associated with the detection of specific responses to weak cellular antigens whose physico-chemical nature is largely unknown and may be unique to individual tumours.

Howie and McBride (1982b)have described a simple, sensitive and reproducible in vitro assay system based on those previously described for soluble hapten-carrier antigens which bypasses the need to measure anti-tumour responses directly. They demonstrated that tumour-specific T_H cells obtained from the spleens of tumour-bearing animals could collaborate in vitro with hapten-primed B cells to generate an anti-hapten antibody response in the presence of haptenated-tumour. The T_H responses thus generated followed the same rules as soluble hapten-carrier responses i.e. the responses show T_H cell specificity for individual tumours and require the hapten to be physically linked to the appropriate tumour cell carrier.

The system developed by Howie and McBride (1982b)was used to investigate the accessory cell activity of tumour-associated macrophages in the expression of tumour-specific T_H cell function.

Cultures containing 10^6 nylon wool-passed spleen cells from an animal bearing a day 14 FSA-R tumour, 5 x 10^6 whole normal spleen cells and 10^5 TNP-FSA-R generated a large anti-TNP PFC response (Figure 13). If accessory cells were removed by passage of the whole normal spleen cell suspension through a Sephadex G-10 column, the anti-TNP response was greatly depleted (Figure 13). This depleted response could however be reconstituted by the addition of accessory cells and tumour-associated macrophages could perform this accessory cell function (Figure 13).

It is clear from the data so far discussed that macrophages only reconstitute Sephadex G-10 depleted PFC responses over a narrow range of concentrations. Optimal responses were normally found in cultures which received around 2% macrophages (resident peritoneal or tumour-associated) and if more than this number of cells were added, then the response generated was considerably reduced. This phenomenon can be seen most clearly in Figure 12.

Niederhuber et al. (1979) proposed that the reduction in the PFC response generated in the presence of high concentrations of macrophages was the result of macrophage-mediated suppression. However, in the present study the addition of adherent resident peritoneal cells to cultures of whole normal spleen cells and CRBC did not significantly alter the magnitude of the anti-CRBC

PFC response generated even at the highest concentration examined (Figure 14). Furthermore, instead of suppressing the anti-CRBC PFC response of whole normal spleen cells, the addition of tumour-associated macrophages actually caused a dose-dependent increase in the magnitude of the response generated (Figure 15).

The enhancing cells present within the tumour-associated macrophage population were further characterized. It was found that although treatment with anti-Ia serum plus complement effectively depleted Ia-positive tumour-associated macrophages (Table XII), this procedure had no significant effect on the ability of these cells to enhance the primary anti-CRBC PFC response of whole normal spleen cells (Figure 16). It may be concluded therefore that only Ia-negative tumour-associated macrophages are involved in enhancement. This conclusion is supported by the finding that although macrophages obtained from tumours grown in nu/nu animals lacked detectable I-A^k antigen (Table IX) they were nevertheless as efficient as macrophages obtained from tumours grown in nu/+ animals at enhancing the primary anti-CRBC PFC response of whole normal spleen cells (Figure 17).

Separation of enzymatically disaggregated tumour cells into populations of differing modal size by centrifugal elutriation (Figure 19; Table XIII) enabled the physical characteristics of the enhancing macrophages to be defined. Correlation

of the degree of enhancement obtained, with the macrophage content of each fraction revealed that enhancement was primarily a function of large macrophages (Table XIII; Figure 20).

Macrophages are known to secrete a number of soluble factors, (monokines), which may stimulate or inhibit lymphocyte responses (reviewed by Unanue, 1981). The possibility was therefore considered that enhancement of the primary anti-CRBC PFC responses of whole normal spleen cells by tumour-associated macrophages was mediated by soluble factors. The results presented in Figure 21 show that culture supernatants conditioned by tumour-derived adherent cells for 24 or 48 hours were indeed highly efficient at enhancing the PFC response.

At least four biochemically distinct antigen non-specific factors have been shown to enhance anti-heterologous-erythrocyte PFC responses. These factors are the T cell-derived lymphokines IL-2 and T cell-replacing factor (TRF), the macrophage-derived monokine IL-1, and CSF, which is produced by several diverse cell types including lymphocytes, macrophages and fibroblasts (reviewed by Farrar and Hilfiker, 1982). In addition, Howard et al. (1981) have suggested the existence of another distinct T cell-derived factor known as B cell growth factor (BCGF), although as yet there is no published biochemical data to distinguish it from any of the other factors.

One of the first clear demonstrations that macrophage-derived soluble factors could enhance in vitro antibody responses came from the studies of Wood and co-workers (Wood and Gaul, 1974; Wood and Cameron, 1975, 1976; Wood et al., 1976). In these studies, medium conditioned by endotoxin-stimulated human monocytes was found to significantly enhance the anti-CRBC PFC response of whole or T cell depleted murine spleen cells. The monocyte-derived factor responsible for enhancement had a molecular weight of approximately 15,000 daltons and was designated B cell-activating factor (BAF) (Wood and Cameron, 1976). Subsequent studies by Unanue et al. (1976) demonstrated that a functionally equivalent factor was also produced by murine peritoneal macrophages.

Although BAF was originally believed to be distinct from other monokines (Wood and Gaul, 1974) studies on the physical and biochemical properties of the factor (Wood, 1979a) revealed a striking similarity to the previously described thymocyte proliferative monokine known as lymphocyte-activating factor (LAF) (Gery et al., 1971, 1972; Gery and Waksman, 1972; Gery and Handschumacher, 1974; Blyden and Handschumacher, 1977) now designated IL-1 (Mizel and Farrar, 1979). This similarity suggests that these activities are either properties of a single molecule or of a number of closely related molecules. In support of the identity of BAF and IL-1, Koopman et al. (1978) and Wood (1979b) both found that BAF activity in the 12,000-18,000 dalton

fraction of supernatants from unstimulated human monocytes could not be separated from the thymocyte mitogenic activity in the same fraction. Similarly, in chromatographic studies, murine macrophage-derived B cell stimulatory molecules were usually found in the same position as IL-1 (Calderon et al., 1975; Mannel et al., 1981). Finally, it is interesting to note that the same stimuli that induced peritoneal macrophages to secrete IL-1 also increased B cell stimulatory activity (Unanue et al., 1976).

Although little disagreement now exists concerning the identity of BAF, the nature of the target cell both in vivo and in vitro remains controversial. Two distinct, although not mutually exclusive views have been expressed. Several groups favour the view that IL-1 acts directly on B cells increasing their proliferation and/or differentiation (Wood and Cameron, 1976; Hoffman, 1979; Falkoff et al., 1983; Lipsky et al., 1983). However, by definition, IL-1 is also able to augment the proliferation of antigen- or mitogen-stimulated T lymphocytes (Mizel and Farrar, 1979) and this activity could lead indirectly to enhanced antibody responses. Thus in mixed cell cultures IL-1 could perhaps produce the same end result by acting on either T cells or B cells. However, Farrar and Koopman (1979) have recently shown that a partially purified preparation of human IL-1 (devoid of IL-2) was able to enhance the PFC response of nude mouse spleen cells only at concentrations

that were supraoptimal with regard to the induction of thymocyte proliferation. It was argued on the basis of these results that T cells express high affinity receptors for IL-1 and can thus be induced to proliferate by physiological concentrations of IL-1. B cells on the other hand were assumed to have low affinity receptors and proliferation could only be induced by very high concentrations of IL-1 which might never be realised in vivo (Farrar and Hilfiker, 1982). It is most likely therefore that the major target cell for IL-1 in the antibody response to thymus-dependent antigens is the T cell, although the possibility that B cells might also be stimulated directly cannot be excluded.

CSF has also been shown to possess enhancing activity. Rubin (1979) demonstrated that a partially purified preparation of murine spleen cell-derived CSF could enhance the PFC response of B cell-enriched murine spleen cells. However this rather crude CSF preparation undoubtably contained various other factors and the importance of CSF is difficult to evaluate. Recently however, Moore et al. (1980, 1981) have demonstrated that preparations of murine fibroblast-derived CSF which were apparently devoid of IL-1, IL-2 and TRF activity were able to enhance the anti-CRBC PFC response of normal murine spleen cells.

A large body of evidence indicates that the primary target cell of CSF is the macrophage (reviewed by Moore et al., 1981). Thus CSF has been shown to be capable of inducing macrophages to

produce secondary mediators such as interferon (Hilfiker et al., 1981), plasminogen activator (Lin and Gordon, 1979), prostaglandins (Kurland et al., 1979) and IL-1 (Moore et al., 1980). The ability to augment the production of IL-1 probably explains the ability of CSF to enhance the PFC response of normal spleen cells.

In the present study, the magnitude of the anti-CRBC PFC response, generated by whole normal spleen cells, was enhanced in a dose-dependent fashion by the addition of tumour-associated macrophages (Figure 15) or nylon wool-passed CRBC-primed spleen cells (Figure 8). The striking similarity in the dose-response curves obtained using these two distinct populations of cells, raised the possibility that tumour-associated macrophages might mediate their enhancing activity by effectively expanding the number of CRBC-specific T_H cells present in the cultures. It was further argued that if this was the mechanism of macrophage-mediated enhancement, then in cultures where T_H cells are in excess, addition of tumour-associated macrophages should have no further effect. If the macrophage-derived factor operated instead of augmenting B cell proliferation and/or differentiation then addition of tumour-associated macrophages would be expected to enhance the PFC response of cultures which contain excess T_H cells. The results of such an experiment are shown in Figure 22. It is clear that while tumour-associated macrophages enhanced

the primary anti-CRBC PFC response of whole normal spleen cells, they had no significant effect on the response of cultures which also received excess nylon wool-passed CRBC-primed spleen cells. Thus it seems that the soluble enhancing factor produced by tumour-associated macrophages does not act directly on B cells. Instead, it is likely that this factor indirectly enhances antibody responses by augmenting the proliferation and / or differentiation of antigen-stimulated T lymphocytes.

Insufficient evidence is available at present to precisely define the nature of the tumour-associated macrophage-derived enhancing factor. However it is evident that although tumour-associated macrophages reconstituted the anti-CRBC PFC response of Sephadex G-10-passed spleen cells (Figure 5), the maximum response generated was not significantly higher than that obtained with whole normal spleen cells. This result suggests that the enhancing factor produced by tumour-associated macrophages does not act directly on T cells, instead it seems that a third cell type which is presumably removed by Sephadex G-10 is involved. Although further studies are clearly necessary it is possible that the enhancing factor could be CSF. CSF is produced in large amounts by "activated" macrophages (Chervenick and LoBuglio, 1972) and is known to indirectly augment T cell proliferation by inducing other macrophages to release IL-1 (Moore et al., 1980). In order to explain the lack of enhancement found in cultures of Sephadex G-10-passed spleen cells and tumour-associated macrophages it is

necessary to assume that tumour-associated macrophages are themselves non-responsive to CSF. In this connection it is interesting to note that macrophages differ greatly in their expression of receptors for CSF (Chen and Lin, 1982). This model can also be used to explain the reduction in the PFC response generated by Sephadex G-10-passed spleen cells in the presence of high concentrations of tumour-associated macrophages (Figure 15). In addition to enhancing factors, macrophages may secrete molecules which inhibit lymphocyte responses (reviewed by Unanue, 1981). Because Sephadex G-10 passed spleen cells lack the target cell for the macrophage-derived enhancing factors, the activity of inhibitory factors can be more readily seen.

CHAPTER VI - CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

CONCLUSION

Since the beginning of the century a vast amount of effort has gone into trying to elucidate the various diverse and complex interactions involved in the induction, expression and regulation of the immune response to neoplastic cells. Behind much of this work lay the belief that through a better understanding of these interactions, it would be possible to develop useful immunotherapeutic approaches to the treatment of human cancer. However today immunological control of neoplasia through therapeutic intervention is still no more than an attractive and elusive goal. Tumour immunology remains by and large phenomenology. Many observations are made but the relationship between them is largely obscure. Clearly *in situ* tumour immunity is an important and often overlooked aspect of the tumour-host relationship. It cannot be emphasized too strongly that the eventual success of immunotherapy will largely depend upon a thorough understanding of all aspects of the tumour-host relationship including intratumoural events.

Although the functional activity of tumour-associated macrophages is an area of fertile speculation, few studies have examined anything other than the cytotoxic activity of these cells (reviewed by Evans and Haskill, 1983). It was the aim of the present study to investigate the accessory cell activity of the macrophage population associated with the murine FSA-R

tumour. These cells were found to be fully capable of reconstituting the primary anti-CRBC PFC response of Sephadex G-10 passed spleen cells. This function required the presence of I-A^k positive cells, and macrophages treated with anti-Ia serum plus complement, or obtained from tumours grown in nu/nu hosts were inactive. However, several recent studies have demonstrated that it is not merely the presence or absence of Ia but also the number of Ia molecules expressed per cell that determines functional accessory cell activity (Matis et al., 1982; Beller, 1984). Since tumour-associated macrophages are clearly heterogeneous with respect to the amount of I-A^k they express, it would be interesting to separate these cells into populations expressing differing amounts of I-A^k and investigate the ability of these various populations to reconstitute the Sephadex G-10 depleted response.

Tumour-associated macrophages were also able to supply the essential accessory cell activity required for cooperation between antigen-primed T_H cells and normal B cells in the generation of PFC responses in vitro. However, the characteristics of the active accessory cell in this system were not further investigated. It would be interesting to determine whether I-A^k-positive cells were also required for this response.

Finally, large I-A^k-negative tumour-associated macrophages were found to secrete a soluble factor(s) which could enhance

the primary anti-CRBC PFC response of whole normal spleen cells. The possibility that this factor could be CSF has already been discussed and certainly deserved further study. It would be relatively simple to test media conditioned by tumour-associated macrophages for their ability to induce bone marrow-derived macrophage precursors to form colonies *in vitro*. In addition, recently developed monoclonal antibodies against CSF could perhaps be used to deplete the enhancing activity from macrophage conditioned medium or block its effect *in vitro*.

From the results presented in this thesis it would seem that the intratumoural environment does not compromise the ability of macrophages to act as accessory cells in the generation of antibody responses *in vitro*. It should be noted however, that tumours generally contain relatively few B cells and it is unlikely that such a response could actually occur *in situ*. Perhaps future studies should utilize an *in vitro* assay system more relevant to the events which may occur within the tumour mass. The generation of lymphokine responses *in vitro* could perhaps constitute a suitable assay system. This response requires that macrophages present antigen to T cells in association with Ia determinants (reviewed by Unanue, 1981). Lymphokines produced as the result of an interaction between tumour-associated macrophages and T cells could attract macrophages to the tumour site and activate these cells to a cytotoxic state. Such events may have important implications with respect to tumour development and metastasis.

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