PART 2

An investigation into some of the properties of monocytes and macrophages in breast cancer patients and normal subjects.

CONTENTS FOR PART 2

INTRODUCTION

Aim

The literature pertaining to the role of the macrophage in cancer is vast and often contradictory, but three broad conclusions can be drawn. Firstly, macrophages are present in virtually all tumours. Secondly, under certain circumstances macrophages can kill tumour cells, in animal models at least. Thirdly, in established human malignant tumours, the presence of macrophages is not sufficient to inhibit growth.

The aim of the work described in the second part of this thesis is to characterise some of the properties and functions of macrophages associated with breast cancer in the hope that some light may be shed on the apparent inability of these cells to prevent growth and spread of the disease. To place the original work in context, a literature review has been carried out, and this forms the major part of the introduction. It is followed by a discussion which looks at some of the unanswered questions and leads on to the section dealing with the experimental investigations.

LITERATURE REVIEW

The role of the macrophage in cancer

CONTENTS

Introduction

Cells of the mononuclear phagocytic system, usually referred to as macrophages, can be divided into two distinct groups - monocytes and tissue macrophages. The monocyte is the circulating form of the cell which originates in the bone marrow and eventually passes into the tissues to become a true macrophage[1]. Although controversy exists, it is widely believed that tissue macrophages are replaced by monocytes rather than undergoing in-situ mitosis[2].

Apart from this major division into monocyte and tissue macrophage, marked heterogeneity exists within both groups[3]. This heterogeneity applies to virtually all the described properties and characteristics of macrophages - size[4,5], morphology[6], phagocytic capacity[7,8], metabolism[9], lysosomal enzyme content[10], expression of receptors for the Fc portion of IgG[11,12], and expression of Ia antigen[13]. It is therefore probable that fully differentiated macrophages exist as several functional subgroups rather than as a single population, although individual macrophages may be capable of undergoing modulation to a certain extent.

Faced with a multiplicity of markers to identify macrophages, none of which are necessarily specific or unique, the problems involved in studying the role of these cells in cancer are daunting. Nevertheless, a great deal of work has been done, and in the following pages, evidence for the participation of macrophages in the modulation of tumour growth

is examined. Several excellent review articles pertaining to this subject have been published[14-18], and the present intention is to provide a background for the original work described later in this thesis.

Evidence that macrophages are present in tumours

The heterogeneity of the macrophage makes its identification difficult, especially in suspensions from tumours where the morphological similarity between tumour cells and macrophages creates a major problem in recognition. However, by using multiple criteria, it is possible to state with certainty that most tumours do contain macrophages, the criteria employed including adherence to glass or plastic, non-specific esterase activity, phagocytosis, expression of Fc or C_3 receptors and the expression of macrophage-specific antigens detected by anti-macrophage serum[17].

Perhaps the most widely used method of ennumerating macrophages in tumour cell suspensions is to detect receptors for the Fc portion of IgG, which are present on the cell surface[19,20], by using red blood cells coated with specific anti-red blood cell IgG to form erythrocyte-antibody (EA) rosettes. This technique is open to criticism as some workers believe that tumour cells may express Fc receptors[21-25]. In addition, it has been shown that human monocytes can shed Fc receptors in culture[26], raising the possibility of non-specific uptake of these receptors by tumour cells. However, cells bearing Fc receptors are absent from long term in vitro cultures of tumours from both mice[27] and humans[28], but the injection of such cell lines back into animals will restore the Fc receptor-positive population within the tumours[27]. Furthermore, injection of tumour cell suspensions enriched for Fc receptor-

bearing cells fails to produce tumours in mice[29], and experiments using antibodies raised against H2 antigens in specific mouse hybrids have shown that Fc receptor-bearing cells in tumours derived from different hybrids are of host origin [29-31]. The macrophage nature of the majority of Fc receptor-positive tumour-infiltrating cells has been demonstrated by their ability to adhere to plastic, to phagocytose colloidal iron or red cells, and to display non-specific esterase staining[29,32].

Although the evidence that tumour cells lack Fc receptors is persuasive, it must be emphasised that cells other than macrophages have Fc receptors - neutrophils[33], subsets of T-[34,35] and B-lymphocytes[36], platelets[37] and mast cells[38]. However, these cells can usually be differentiated from macrophages on morphological grounds, and they do not present the same difficulties in identification as do tumour cells.

Using a variety of techniques, commonly Fc (IgG) receptor expression, various workers have reported that 0 - 65% of the cells recovered from tumours of different types were macrophages[17]. In human tumours the range is 0 - 56% [39-41], and of particular interest is the study by Wood and others where cell suspensions prepared from four breast carcinomas were found to comprise 27%, 48%, 54% and 55% macrophages respectively[40]. The evidence therefore indicates that most tumours contain substantial numbers of macrophages, and in the ensuing sections, the function and properties of tumour-associated macrophages will be examined critically.

The effect of macrophages on tumours

The study of macrophage effects on tumours is, by necessity, almost exclusively confined to experimental animals. It can be approached in vivo by examining the alteration of tumour growth patterns caused by agents known to modify macrophage function, and in vitro by assessing the function or properties of macrophages associated with tumours and by the use of cytoxicity assays. The term "cytotoxicity" must be interpreted with caution, as it may refer to cytolysis or cytostasis. Tests involving the release of isotope from prelabelled tumour cells (e.g. the ^{51}Cr-release assay) almost certainly measure cytolysis, but growth inhibition tests are less reliable for cytostasis. Cytolysis balanced by cell division may be responsible for apparent growth inhibition[42], and incorporation of labelled DNA precursors into target cells has fallen into disrepute as a measure of cytostasis owing to the discovery that macrophages release a compound which can interfere with their uptake[43]. Despite these problems, however, it is still possible to draw conclusions from many studies regarding the ability of macrophages to subvert tumour growth.

Normal, unstimulated mouse peritoneal macrophages are not cytoxic for tumour cell lines[44,45]. However, these cells can be induced to kill tumour cells in vitro if they are exposed to a variety of factors including BCG[8,46,47], systemic adjuvants[48], chronic toxoplasma infection[49], and bacterial products[50]. In addition, Corynebacterium parvum (C. parvum) has been shown to protect against the growth of a murine

mammary carcinoma[51], and local BCG can increase the macrophage content of transplantable tumours[47,52].

This process of rendering macrophages cytotoxic is termed "activation" and is non-specific in that activated cells will kill a wide range of transformed target cells. It is possible, however, to produce "immune" macrophages by exposing mice to a tumour cell load, and adherent peritoneal cells from such animals will display in vitro cytotoxicity which is specific for the immunising cell line[44,45,53]. This phenomenon appears to require the participation of lymphocytes[44], and it has been shown that peritoneal macrophages from non-immune mice can be induced to kill specific target cells by a factor released from immune lymphocytes incubated with the tumour cells in question[54-56]. This factor is called "specific macrophage arming factor", and is derived from T-lymphocytes.

It is clear, therefore, that at least two distinct sets of conditions are required for cytotoxicity - macrophages must be "activated" or "armed" - but the effector mechanisms are not well established. Cell to cell contact appears to be necessary in most systems involving activated[46] and immune macrophages[45,53,56], but phagocytosis does not seem to be involved[53,57]. However, Nelson has pointed out that activated macrophages may utilise different tumouricidal mechanisms in different circumstances: in studies of a mouse fibrosarcoma involving small volumes and cell numbers, release of arginase by effector cells caused tumour cell death, whereas, when larger cell numbers were used, macrophage-target cell contact

was required and arginase was not involved[18]. Macrophage anti-tumour effector mechanisms may also depend on lymphocyte function: there is evidence that C. parvum-activated macrophages may require lymphocyte factors to function as killing cells[51,58,59], and macrophages have been shown to exhibit antibody-dependent cellular cytotoxicity[57,60-63]. Lymphocyte participation in macrophage-tumour interaction is dealt with in more detail later (pages 20 - 25).

The evidence presented above gives ample reason to believe that macrophages can kill tumour cells under certain in vitro conditions - the important question to answer is whether they do so or are capable of doing so in vivo. Peritoneal macrophages from adult mice, which have a high spontaneous resistance to a transplantable plasmacytoma, confer a similarly high resistance on newborn mice which are usually highly susceptible to this tumour[64], and innoculation of activated macrophages into tumour-bearing rats consistently diminished neoplastic growth[65]. This suggests that macrophages can kill tumour cells in vivo, and is supported by the finding that anti-macrophage agents such as silica and carageenan can promote tumour growth[65] and abrogate the anti-tumour activity of activating agents such as BCG[66] or C. parvum[51,66].

Having established that macrophages can have some tumouricidal effects in vivo, it is important to consider the actual tumour-infiltrating macrophage. A purely scavenging role for these cells seems unlikely, as no relationship exists

between macrophage content and tumour necrosis[67]. The macrophage content of transplantable tumours has been reported to be inversely related to the number of neoplastic cells[32], but a positive correlation between macrophage content and tumour size has also been demonstrated[68], and yet others have found no relationship between tumour dimensions and macrophage infiltration[67,69]. However, regressing tumours have consistently higher percentages of macrophages than progressing tumours, regardless of whether regression is induced[52,70] or spontaneous [71-73], and there is some evidence that metastases contain relatively fewer macrophages than do their primaries[39]. This might suggest that tumour-infiltrating macrophages are responsible for in-situ tumour cell destruction, but macrophage content does not affect neoplastic growth rates[30], nor can it predict whether a given tumour will progress or regress[70,74].

Another approach is to study the effect of macrophages on the ability of tumours to metastasise, and this has been done by comparing experimental tumour systems of differing immunogenicity, rather than by observing the variation of metastatic behaviour within a single spontaneous tumour type. The early work in this field suggested that macrophage content was directly related to immunogenicity and inversely related to metastasising tendency[31,75,76]. Also, ultrastructural studies of hamster tumours indicated that phagocytic cells in the metastasising variety had more prominent phagosomes[77]. However, more recent research has failed to demonstrate a relationship between tumour macrophage content and immunogenicity[78,79] or metastatic potential[80], and Evans has suggested that the small

numbers of tumours in the original studies may have been responsible for inaccurate conclusions[79]. Nevertheless, the possibility remains that macrophage content may be important in determining metastatic behaviour within individual tumours. One series of experiments has demonstrated that depletion of adherent cells from fibrosarcoma cell suspensions increases the likelihood of metastatic disease following injection into mice[29], although this might merely reflect an increased tumour cell burden. Another study, ennumerating macrophages on tissue sections of human breast cancers by histochemical identification of acid phosphatase, suggests that macrophage content may be related to prognosis[81]. However, this finding may relate to a small subgroup of acid phosphatase containing cells, and may not indicate the effect of total macrophage content.

Another important factor is the functional ability of macrophages within tumours, because, although non-tumour derived macrophages can kill neoplastic cells, and macrophage content can be correlated with some aspects of tumour behaviour, the fact remains that the presence of substantial numbers of macrophages in a tumour does not prevent progressive growth. It is certainly possible to isolate macrophages which are non-specifically cytotoxic in vitro from a variety of tumours[4,5,74,76,82], and non-cytotoxic macrophages from progressively growing mouse tumours can respond to small quantities of bacterial lipo-polysaccharide by developing high levels of non-specific cytotoxicity - a property not shared by non-activated adherent peritoneal cells from tumour-bearing animals[83]. In addition,

a subpopulation of macrophages from an immunogenic murine fibrosarcoma has been shown to express increased Fc receptor expression when compared to non-activated peritoneal macrophages [84]. This evidence would suggest that, although established tumours do not contain specifically immune mononuclear phaogcytes, they can harbour activated macrophages. Of course, the activated state can be demonstrated functionally only after the cells have been isolated, and the tumour environment may well suppress any inherent cytotoxic ability these macrophages might have. Such a view is supported by the finding that established tumours in mice form a privileged site for the growth of listeria monocytogenes[85], since the transfer of tumour immunity by T-cells confers non-specific activation on macrophages which is paralleled by a powerful macrophage-mediated resistance to this bacterium[86].

In the clinical setting, macrophage activation is an attractive concept to the cancer therapist, and clinical trials employing activating agents have been carried out[87]. These studies have been rather disappointing, although one, performed at Rosewell Park, indicated significantly improved survival in advanced lung cancer patients receiving C. Parvum plus chemotherapy compared to those having chemotherapy alone[88]. However, a trial of levamisole in patients with operable breast cancer suggested that this adjuvant produced a deleterious effect in terms of disease recurrence rates[89]. Levamisole can restore to normal the functions of phagocytes and T-lymphocytes from compromised hosts[90], but, in defense of rational immuno-therapy, it should be mentioned that the compound has been shown

to decrease the non-specific tumouricidal ability of mouse peritoneal macrophages[48].

Finally, it is important to consider the possibility that macrophages might stimulate tumour growth. Keller has suggested that enhancement of tumour growth by anti-macrophage agents might be due to the release of growth-promoting substances from the damaged cells[65], and Evans has shown that culture supernatants from either cytotoxic or non-cytotoxic tumour macrophages could stimulate non-specific proliferation of neoplastic cells in vitro[91]. Evans has also demonstrated that slowing or cessation of murine fibrosarcoma growth by irradiation is paralleled by a decrease in macrophage infiltration, and that resumption of growth is accompanied by an increase[92]. Whether this represents cause or effect is uncertain, but, using the same model, Evans found that injection of thioglycollate-stimulated or transformed peritoneal macrophages along with tumour cells into mice led to stimulation of tumour growth, and reconstitution of X-irradiated mice using bone marrow cells allowed normal tumour growth, and restored the level of macrophage infiltration to near-normal levels[93]. Further incriminating evidence comes from a recent study in which transferred thioglycollate-elicited peritoneal cells were found to increase the number of metastatic lung nodules produced by intravenous injection of tumour cells into mice[94].

It appears, therefore, that under certain conditions, macrophages can stimulate tumour growth and may be required

for normal tumour progression. How general or idiocyncratic these findings are is, as yet, not known, and the mechanisms by which tumour enhancement may take place are equally obscure. Again, lymphocytes may be involved, but this will be discussed in a later section (pages 20 - 25).

The effect of tumours on macrophages

In certain murine systems, the presence of established tumours can lower the ability of the host to reject a subsequent tumour challenge, particularly in the early phase of growth[95,96]. This inhibition of tumour resistance is paralleled by depression of macrophage-mediated antibacterial activity against listeria monocytogenes and Yersinia enterocolitica, suggesting that the escape from anti-tumour resistance may represent subversion of macrophage function by the tumour[95]. Further evidence that neoplasia can compromise macrophage function is provided by the finding that the ability of animals to mobilise macrophages to an inflammatory site is markedly depressed by tumour implantation, and that invitro chemotaxis of peritoneal macrophages is similarly susceptible[97-99]. These effects can be reproduced by tumour cell supernatants[96,100] or serum from tumour-bearing animals[100], and the inhibitory factor has been identified as a low molecular weight heat stable molecule[96]. There is tentative evidence that tumour cells may also secrete factors which prevent contact between themselves and macrophages[101], and tumour innoculation can reduce the rate of colloidal carbon clearance from the circulation, suggesting an inhibitory effect on phagocytosis[100]. In addition, regional lymph nodes from tumour-bearing areas in mice accumulate radio-labelled colloid less readily than normal nodes, a finding which lends further credence to the view that tumour factors may inhibit phagocytosis[102].

In humans too, there is evidence that neoplasia

has adverse effects on macrophages. The presence of malignant disease is associated with defective monocyte chemotaxis[103-105], and the maturation of monocytes to macrophages appears to be depressed by serum or soluble tumour extracts from patients with squamous carcinoma of the lung[106]. Phagocytic function may also be affected, because the clearance of radiolabelled aggregated albumin is lowered in patients with metastatic disease, although it is actually high in those with localised malignant tumours[107]. Furthermore, the observation that regional lymph nodes draining tumours have depressed ability to take up colloid has also been made in breast cancer patients[108], although not all workers endorse this finding[109].

Macrophage surface receptors may also be modified by tumours, and, in a series of careful experiments, Rhodes has shown that human tumour supernatants can lower the expression of Fc receptors by adherent blood monocytes, and that pulmonary alveolar macrophages from the vicinity of bronchogenic carcinoma have less Fc receptor activity than similar cells from non-tumour patients[110,111]. Paradoxically, blood monocytes from cancer patients have higher levels of Fc receptor expression than comparable controls[112], but this will be discussed in a later section (pages 26-30).

Although tumours can certainly depress aspects of macrophage function, they can also have stimulatory effects. They clearly have the ability to accumulate a macrophage population, and there is evidence from experimental systems that previously activated radiolabelled macrophages may be selectively attracted[113]. Tumours may also stimulate

proliferation of mononuclear phagocytes; the presence of both animal and human neoplasms is associated with a monocytosis[114-117], the process of stem cell migration from bone marrow is increased in tumour bearing mice[118], and macrophage colony formation from marrow cells is enhanced during the initial growth of a transplantable murine mammary tumour[119]. Stimulation of peritoneal macrophages as reflected by increased DNA synthesis is also a feature recorded in mice with actively enlarging tumours[120].

Apart from these proliferative phenomena, neoplasia may produce other stimulatory effects in macrophages. As mentioned above, monocytes in cancer patients have increased Fc receptor expression[112], and lysis of antibody-treated erythrocytes by blood monocytes is more prominent in patients with malignant melanoma[121]. Strangely, one study has demonstrated increased phagocytosis of IgG-coated red cells by peritoneal macrophages in tumour-bearing mice[98], which is at variance with the work involving colloid. However, this may reflect increased Fc receptor activity rather than an increased phagocytic capacity, and it should be mentioned that these same macrophages displayed markedly depressed chemotactic response.

The mechanisms underlying these tumour-associated events are enigmatic, but prostaglandins have been widely implicated as possible mediators in tumour resistance against the host response. Many tumours and neoplastic cell lines have been shown to produce these compounds[122-125], and

prostaglandins of the E series are capable of inhibiting the tumouricidal and tumouristatic effects of activated mouse peritoneal macrophages[126,127]. The role of prostaglandins is far from clear, however, as normal macrophages may be induced to develop anti-tumour activity by prostaglandins[127]. The source of macrophage-modulating factors from tumours is also a subject for debate, as macrophages themselves form a major source of prostaglandins[128-130], and, as discussed in the next section, tumour-associated lymphocytes may be extremely important in altering the behaviour of mononuclear phagocytes. Finally, the mode of action of tumour derived factors is difficult to assess, and it is possible that substances emanating directly from tumour cells have their effect on macrophages via lymphocytes - a subject reviewed in the next chapter.

Interaction between macrophages and lymphocytes

From the preceding section, it is evident that under various conditions, macrophages can destroy or support tumour cells, and tumours can either depress or stimulate macrophages. As macrophage function is closely linked with that of lymphocytes[131], it is important to consider how interaction between these two cell types might be implicated in the overall effect.

Firstly, the accumulation of macrophages in tumours may be lymphocyte dependent. Neoplasms in athymic nude mice or in mice depleted of T-cells by thymectomy or thoracic duct drainage have been shown to have low levels of macrophage infiltration[72,75], and immunosuppression by anti-lymphocyte or anti-T cell serum has a similar macrophage depleting effect [27,132]. This is not a universal finding, however, and at least one group have failed to demonstrate this phenomenon[32].

Lymphocytes have also been implicated in the production of macrophage-mediated cytotoxicity, and, as previously mentioned, immune macrophages appear to have their specific cytotoxicity conferred upon them by a lymphocyte derived factor[44,54,55]. This "Specific Macrophage Activating Factor" does not appear to be immunoglobulin, since treatment of monolayers of armed mouse macrophages by anti-mouse gamma-globulin does not affect specific killing[56]. Antibody does seem to have a role to play, however. It has been shown that the ability of C. parvum-activated macrophages to protect against a transplantable murine carcinoma in vivo closely

parallels the ability of the host to mount a humoural response to sheep erythrocytes[51], although it must be borne in mind that such a relationship may be entirely fortuitous. More convincingly, several groups have adequately demonstrated that macrophages can display antibody-dependent cellular cytotoxicity[57,60-63], and there is evidence that macrophage-bound immunoglobulin may mediate the presentation of antigen to T-lymphocytes[133-135]. Both of these mechanisms may be important in tumour cell killing.

"Activated" or non-specifically cytotoxic macrophages may also be modulated by lymphocytes. BCG-activated macrophages lose their tumouricidal ability after in vitro culture, but this has been shown to be retained if lymphokine is present along with tumour antigen[58] or lipopolysaccharide[59]. In addition, the cytotoxicity of interferon-activated macrophages can be enhanced by supernatant from lymphoid cells stimulated by phytohaemagglutinin or C. parvum[136], and the transfer of tumour immunity by T-cells from mice with sarcomas is paralleled by non-specific activation of macrophages[86].

Just as macrophage function may be triggered or enhanced by lymphocytes, the reverse situation can also hold. Adherent spleen cells from mice, and monocytes from humans can increase the lymphocytic response to phytohaemagglutinin [137,138], and BCG-activated peritoneal macrophages display this ability to a greater extent that untreated cells[139]. T lymphocytes produce a factor which has similar effects[140], and it is now known that the lymphoproliferative effect of macrophage-produced "Lymphocyte Activating Factor" (now known

as Interleukin 1) is mediated by stimulation of the release of "T-cell Growth Factor" (Interleukin 2) from T-lymphocytes [141,142]. Another pivotal function of the macrophage is the presentation of mitogens and soluble antigen to T-lymphocytes [143-145], but, as with the interleukin mechanism, its importance in tumour immunity is uncertain, although presumably both would be critical in the production of a T-cell mediated tumour-specific allergic response.

Also relevant to the tumour situation is the effect of macrophages on NK (Natural Killer) activity. The NK cell is a lymphocyte which displays cytotoxicity against a variety of target cell lines, including neoplastic cells, but which is neither specifically immune nor dependent upon antibody[146]. Macrophages seem to play a role in regulating NK cells, as they enhance NK activity in hamsters[147] and mice[148,149].

Lymphocyte function may also be suppressed by macrophages[150], and this is of particular interest as, in some situations, the macrophage appears to be advantageous for tumour growth (q.v. pages 14-15). In rats, various tumours depress the host's ability to produce a delayed hypersensitivity response, and this is most pronounced in tumours with high macrophage contents[151]. NK cells may also be subject to suppression by macrophages, and in contrast to the studies cited earlier, NK activity has been shown to be depressed by macrophages in mice and humans[152,153]. Furthermore, in mice bearing tumours, marked macrophage-dependent suppressor activity affecting T and B lymphocyte responses to mitogens and T-dependent mixed leukocyte reactions has been observed[154].

However, a recent study of macrophage-mediated suppression has demonstrated an inconsistency between in vitro and in vivo findings[155]. Growth of a spontaneous carcinoma in mice was found to cause a progressive macrophage-mediated hyporesponsiveness of spleen cells to phytohaemagglutinin and to the mixed leukocyte reaction, but tumour-bearing mice displaying this depressed T-cell reactivity in vitro were able to reject an allogeneic mastocytoma to the same extent as normal mice. This ability to reject the mastocytoma was abrogated by irradiation and recontituted by normal T-cells in either normal or tumour-bearing mice, suggesting that in vitro suppression may not reflect the in vivo situation.

The mechanisms whereby macrophages have their effects on lymphocytes are not clear, but two substances have been particularly implicated - prostaglandins and interferon. Macrophages undoubtedly produce prostaglandins[128,156,157], and their role in immunoregulation appears to be predominantly one of suppression[130]. Prostaglandins, particularly those of the E series, can inhibit antibody formation, mitogen stimulation of lymphocytes, and the delayed hypersensitivity reaction[158-160]; involvement of mononuclear phagocytes in prostaglandin suppressor systems is suggested by the findings that macrophage-mediated depression of B-lymphocyte proliferation and macrophage activation of T-suppressor cells can be abrogated by prostaglandin synthesis inhibitors[156,161]. Furthermore, NK activity can be inhibited by prostaglandin E[149,162,163], and lymphocyte traffic through antigen-stimulated lymph nodes appears to be mediated by the same molecule[164].

In tumours, however, the picture is less well defined, because, although many tumours contain high levels of prostaglandin[122-125], it is not clear whether it originates from tumour cells or host cells. Certainly, splenic and peritoneal macrophages from tumour-bearing animals produce more prostaglandin E_2 than do macrophages from normal animals[157], but the situation within the tumour might be quite different. The actual effect of prostaglandin in the tumour situation is also obscure; prostaglandin synthesis inhibition can reverse tumour-induced immunosuppression and retard tumour growth in mice[165], so it might be thought that prostaglandins might mediate macrophage-induced tumour growth. However, prostaglandin synthesis inhibitors have also been shown to enhance tumour growth, and, conversely, prostaglandin E may suppress it[166], and this paradox is not yet resolved.

The second mediator, interferon, seems to have a stimulatory effect. Mouse NK activity is increased by interferon or by poly 1:C (an inducer of interferon), and the augmentation caused by poly 1:C can be prevented by the removal of macrophages[148]. However, after induction of interferon by poly 1:C, removal of macrophages had no effect, and after removal of these cells, interferon was still capable of increasing NK activity. This would suggest that macrophages augment NK activity by producing interferon, but they may also depress it by means of a prostaglandin, and one study has demonstrated that macrophage-mediated NK augmentation is increased by prostaglandin synthesis inhibition, indicating that a dual regulating system may operate[149]. However, in

hamsters, cell to cell contact appears to be necessary for peritoneal macrophages to stimulate NK activity in bone marrow cells[147], and in humans, peripheral blood lymphocytes only showed NK activity after removal of adherent monocytes[153]. There are either major species differences, or the interaction between macrophages and NK cells varies greatly according to the in vitro conditions of the different assays, or the anatomical origin of the lymphoreticular cells.

In summary, lymphocytes may help macrophages to produce anti-tumour effects by inducing accumulation of macrophages within tumours, by specifically arming macrophages, by producing antibody to mediate antibody-dependent cellular cytotoxicity or by enhancing the non-specific cytotoxicity of activated macrophages. Mononuclear phagocytes may also operate through lymphocyte effector mechanisms by stimulating or suppressing lymphocyte functions, and the possible role of these mechanisms in tumour immunity is best exemplified by natural cytotoxicity.

The role of macrophage surface characteristics

i) The Fc (IgG) receptor

Many of the cells involved in host defense mechanisms bear surface receptors for the Fc portion of IgG. This applies to neutrophils[33], some T-lymphocytes[34,35] and some B-lymphocytes[36,167], but the Fc receptor is most consistently seen on cells of the mononuclear phagocyte series[2,19,20]. Consequently, Fc receptor expression, detected by means of IgG-coated erythrocytes, has been used to identify macrophages in cell suspensions from tumours[17], but this may be less than satisfactory for two reasons. Firstly, some workers believe that tumour cells may display Fc receptors, and although the evidence against this is persuasive (q.v. pages 6 - 7), the possibility cannot be entirely discounted. Secondly, macrophages exhibit a marked heterogeneity in Fc receptor expression[11,12], and it must be borne in mind that when only Fc receptor-bearing cells are considered, an undefined population of macrophages lacking Fc receptors may also be present.

In addition to true heterogeneity, macrophages may also display some plasticity of Fc receptor expression. For example, when human monocytes are cultured to allow differentiation into macrophages, increased Fc receptor expression is seen[168]; this may be due to increased numbers of Fc receptors, to increased avidity of the receptor for IgG, or to more complex mechanisms such as differences in receptor mobility. Despite

uncertainty as to the true nature of changes in Fc receptor expression, however, the phenomenon has attracted a good deal of interest. In a study using IgG-coated erythrocyte (EA) rosettes, breast cancer patients were shown to have a higher percentage of Fc receptor-positive mononuclear cells in their peripheral blood than did healthy controls, although no distinction was made between macrophages and lymphocytes[169]. Using a range of IgG concentrations to produce EA indicators, Rhodes has developed a more sensitive technique whereby the Fc-receptor expression of adherent monocytes or macrophages can be estimated using a dose-response curve to indicate the avidity of the cells for the indicators[11]. Employing this method he demonstrated that blood monocytes from humans with solid malignant tumours had increased Fc receptor activity[111,112,138], but that pulmonary alveolar macrophages from lungs bearing bronchogenic carcinoma had depressed Fc receptor expression when compared to similar cells from patients without tumours[111]. This depression was unlikely to be due to receptor blockade by IgG or immune complexes, because trypsin treatment, which can remove surface-bound molecules[170], did not alter the difference between the two groups[111]. In addition, supernatants from human solid tumours were shown to diminish Fc receptor expression on human monocytes[110,111] and, paradoxically, the addition of normal human serum to monocytes also depressed Fc receptor expression whereas the addition of tumour patients' serum had the opposite effect[110]. This state of affairs may be explained by an Fc receptor-suppressing

or blocking factor which is present in normal human serum, but which is sequestered by tumours. The identity of this factor remains obscure, but endogenous retinoids, which can depress Fc receptor expression[171] and are found within tumours[172] have been forwarded as a possible candidate.[111]

Despite Rhodes' work, however, it has yet to be demonstrated conclusively that tumour-infiltrating macrophages do have depressed Fc receptor activity, and at least one study has suggested that this might not be the case[84]. Subpopulations of macrophages from a murine fibrosarcoma were separated by unit gravity velocity sedimentation, and Fc receptor expression analysed using a method similar to that of Rhodes. Of the two populations obtained, the smaller macrophages were found to have similar Fc receptor activity when compared to peritoneal macrophages elicited by protease peptone from the same mice, and the larger tumour-infiltrating macrophages actually had increased receptor activity[84]. This would suggest that, under some circumstances at least, macrophages from tumours can display enhanced Fc receptor expression.

In view of these findings, it would be of great interest to know whether Fc receptor expression in any way parallels anti-tumour activity. Stimulation of rabbit alveolar macrophages by multiple intravenous injections of complete Freund's adjuvant leads to an increase of Fc receptor expression [173], and injections of mineral oil have the same effect on guinea pig peritoneal cells[11]. Additional evidence comes from a study which indicated that BCG could protect the Fc rosette forming ability of mouse peritoneal macrophages from the

inhibiting effect of antisera directed against the major histocompatability complex[174]. These findings would suggest that Fc receptor expression may be a measure of macrophage activation, and this view is further supported by the discovery that interferon, a known macrophage activating agent[136,175], can increase the rate of phagocytosis of IgG-coated erythrocytes by mouse macrophages[176], although whether this is due to an alteration in Fc receptor avidity or a direct effect on the phagocytosis mechanism is not clear.

The Fc receptor may also be an integral part of the anti-tumour mechanism of the macrophage. As mentioned earlier (q.v. page 21), macrophages are capable of displaying antibody-dependent cellular cytotoxicity, demonstrated in vitro using red blood cells[57,60], or tumour cells[61-63], as targets. The actual killing mechanism in this process does not involve phagocytosis[57] and indeed, cytochalasin B, which inhibits macrophage phagocytosis, actually enhances cytotoxicity[60]. It is also possible that macrophage-bound antibody might mediate the presentation of antigen to T-cells[133,134], and this may be involved in the generation of a tumour-specific allergic response. If antibody-dependent macrophage-mediated cytotoxicity or cytophilic antibody-associated antigen presentation do constitute important mechanisms in tumour resistance, then the Fc receptor must certainly be critical in its expression. This would confer great significance on alteration in Fc receptor expression in the tumour environment, and might also offer an explanation as to how tumours escape

host-induced destruction. It is known that cancer patients have high levels of circulating immune complexes[177,178], and IgG complexes are fixed to macrophages via the Fc receptor[12]. The receptor has a greater affinity for antibody-antigen complexes than for antibody alone[179], and if the macrophage encounters soluble complexes before assuming the role of the effector cell in antibody-dependent cellular cytotoxicity at the tumour site, its efficacy in this capacity will be reduced.

ii) The C3 receptor

C3, a component of the complement system, has multiple functions in host defense. It exists in plasma and other fluids in inactive form and is cleaved by C3 convertase ($C\overline{24}$) to form C3a and C3b[180,181]. C3a is an anaphylotoxin which causes the release of histamine from mast cells[180]. It is also chemotactic for neutrophils and it causes contraction of smooth muscle[180,181]. C3b binds to the surface of cells[182] and other particles inducing immune complexes to facilitate ingestion by phagocytic cells[183,184]. C3 is also central to the complement system itself, as it modulates several enzymes of the classical and alternative pathway[185].

C3 receptors were recognised on human monocytes by virtue of the fact that they could bind IgG-coated erythrocytes incubated with C1, 2, 3 and 4 in the presence of

free fluid phase IgG which inhibited binding in the absence of complement[186]. In addition, IgM-coated red cells do not normally bind to monocytes, but will do so if incubated with C1, 2, 3 and 4[186]. These erythrocyte-antibody-complement (EAC) indicators made using IgM constitute a useful method of detecting C3 receptors, and rosetting reactions have indicated that C3 receptors are present on neutrophils[187,188], some lymphocytes[189], and on a high proportion of macrophages from animals[188] or humans[2].

The purpose of the C3 receptor on macrophages is open to speculation, but several theories are plausible. It is thought by some that the C3 receptor compensates firstly for the inability of IgM to react with macrophages, and secondly for the blocking of Fc (IgG) receptors by free IgG. The Fc (IgG) receptor, on the other hand, compensates for the lower complement-binding capacity of IgG compared to IgM[186]. In addition, the interaction between C3 and its receptor on the macrophage appears to be a stimulus for various cellular events. Human neutrophils react to non-phagocytosable C3-coated sepharose beads by increasing their oxidative metabolism[190], and the same cells can be induced to release lysosomal enzymes either by activation of the indirect complement pathway[191] or by adherence to a C3-coated surface[192]. By means of chemiluminescence, purified C3b has been shown to increase respiration in human monocytes[193], and it can also stimulate the release of lysosomal enzymes from guinea pig and mouse peritoneal macrophages[194,195].

It seems, therefore, that C3 receptors on macrophages may firstly complement Fc receptors as a mechanism for immune adherence, and secondly, act as a trigger for metabolic events. In tumour immunity, however, the role of the C3 receptor is obscure. Unfortunately, EAC rosettes are not particularly useful for ennumerating tumour-infiltrating macrophages, since trypsin can strip off or interfere with the C3 receptor[186,187], and this enzyme is hard to avoid when disaggregating tumours. Little is known about variations in C3 receptor expression, although one group has reported that the total percentage of C3 receptor-positive mononuclear cells in the peripheral blood of breast cancer patients is not different from that found in healthy controls[169]. Mouse peritoneal macrophages which are not cytotoxic for a mastocytoma cell line may become so when cultured with C3b, the release of C3a being implicated in the effector mechanism[196], and the tumouricidal capacity of activated mouse macrophages has been shown to be trypsin-sensitive[197]. Conceivably, therefore, the C3 receptor may be involved in tumour killing mechanims, but much more work is required to define its role precisely.

iii) <u>The Ia surface antigen</u>

The genetic control of the immune response to polypeptides is effected by genes linked to the transplantation locus of the species. These genes are known as the immune response (Ir) genes, and the region of the major

histocompatibility complex which includes them is termed the "I" region. The surface proteins which are coded in the I region are called immune response associated (Ia) antigens because they were defined using alloantibodies, and, in humans, they correspond to the HLA-D transplantation antigens[131].

These Ia antigens are present on the surface of B lymphocytes, some T lymphocytes and macrophages[131,198,199], and it has been suggested that alteration of Ia determinants by antigen is a stimulatory signal for lymphocyte activation[200]. Certainly, in mice, effective cooperation between macrophages and T cells requires identity at the H_2 I region[201], and the ability of mouse macrophages to present antigen to T cells is blocked by anti-Ia monoclonal antibody, and abrogated by depletion of Ia-positive macrophages[13]. Variations in the expression of Ia antigens certainly occur, and both a fall[13] and a rise[202] have been seen to occur in macrophages or monocytes in culture. However, Ia heterogeneity in the cancer situation has not been widely studied, and the role of the Ia determinants in tumour immunology has yet to be defined.

The role of phagocytosis

Phagocytosis is a property displayed by macrophages from all sites[203,204], and although it can be mediated by Fc or C_3 receptors[205], the participation of IgG or C_{3b} is not mandatory[206]. Despite the use of phagocytosis as a criterion in the definition of the macrophage however, a marked heterogeneity exists, and cells which display other characteristics of mononuclear phagocytes may be incapable of exhibiting this process[7,8]. It is therefore of interest to determine whether tumours can affect phagocytosis, and whether phagocytosis has any part to play in anti-tumour defense mechanisms.

In mice, innoculation of Lewis lung carcinoma cells causes a depression in the rate of clearance of carbon particles from the circulation during the first 72 hours[100], and injection of mammary carcinoma can depress the uptake of labelled antimony sulphate colloid by regional lymph nodes[102]. In humans with cancer the picture is confused, as the rate of clearance of aggregated albumin from the blood has been shown to be high in patients with large non-metastatic tumours, but low in patients with small tumours and nodal tumour spread[107]. However, uptake of colloid by regional lymph nodes in patients with breast cancer appears to be depressed as in the animal experiments[108], although this is not a universal finding[109].

These results are somewhat inconclusive, and carbon clearance studies need not exclusively measure macrophage function[16], but they do suggest that phagocytic function may be depressed in the presence of tumours. This is supported by

the finding that mouse malignant cells can release a compound with phagotoxic effects[207], but at least two other studies would suggest a contrary conclusion. Intravenous injection of brominated fluorocarbon emulsion causes radiopacification of tumours in rodents, and ultrastructural studies indicate that the substance is taken up by macrophages within the tumours suggesting that their phagocytic function remains at least partially intact[208]. Furthermore, phagocytosis of IgG-coated sheep erythrocytes by peritoneal macrophages is increased during the early stages of tumour growth in mice[98] although this may be related to the enhanced Fc receptor activity known to accompany neoplasia[112].

In terms of anti-tumour effector mechanisms, the importance of phagocytosis is doubtful. There is ultrastructural evidence that murine Kuppfer cells may be capable of engulfing tumour cells by surrounding them with cytoplasmic processes[209], but other studies would suggest that phagocytosis is not involved. For example, a subpopulation of adherent, esterase positive murine peritoneal cells, which were isolated on the basis of deficient phagocytosis, were shown to be cytotoxic for various tumour cell lines in vitro[8]. In addition, macrophage-mediated antibody-dependant cellular cytotoxicity against chicken erythrocytes can be enhanced by inhibiting phagocytosis using cytochalasin B[60], and tumour cell cytotoxicity both antibody-dependant and independant has been shown not to involve phagocytosis by other workers[53,57].

Nevertheless, treatment of tumour-bearing mice with C. parvum can lead to an increased rate of colloidal carbon

clearance which is associated with tumour shrinkage[210] and activation of mouse peritoneal macrophages by C. parvum or lipopolysaccharide causes an increased ability to phagocytose IgG-coated red cells[176]. It is therefore possible that the phagocytic capacity of the macrophage, although unlikely to affect its tumouricidal ability directly, may reflect its level of activation.

The role of cytoplasmic factors

i) Enzymes in general

The macrophage has divers functions, and among these, the ability to secrete a wide variety of substances is of major importance. At least 54 secretory products have been described[211], and enzymes make up a substantial proportion of this array. It is likely that proteolytic enzymes have a role to play in the tumouricidal activities of macrophages, as protease inhibitors can prevent macrophage-mediated target cell lysis[197,212], and mouse macrophages have been seen to transfer lysosomes directly into target tumour cells, causing heterocytolysis[213]. Also worthy of note is the finding that thioglycollate stimulation of murine macrophages leads to increased secretion of collagenase[214] and elastase[215,216]. Furthermore, activation of mouse macrophages by BCG or bacterial products causes increased phosphatase activity[48], and intravenous C. parvum in cancer patients significantly increases the numbers of esterase-positive monocytes[217].

It would seem, therefore, that cytoplasmic enzymes in macrophages may be important in tumour killing, and the levels of these enzymes certainly seems to be altered in activation. In the following pages, two cytoplasmic factors - lysozyme and alpha-1-antitrypsin - will be examined in detail, and their possible roles in macrophage-mediated anti-tumour activity will be discussed.

ii) Lysozyme

Lysozyme was discovered and named in 1922 by Alexander Fleming[218], who described it as "a remarkable bacteriolytic element found in tissues and secretions." It is a small molecular weight cationic protein[219], and its main substrate is the bacterial cell wall where it acts through the dissolution of N-acetyl-glucosaminyl-N-acetylmuramic acid linkages[220]. Because of this action, the synonym "muramidase" is often used, but "lysozyme" is the term recommended at an international conference held at Columbia University in 1972[221]. Not all bacteria are susceptible to lysozyme, either because of an impenetrable layer or because of a tightly-woven cell wall structure[219], but other factors can collaberate with the enzyme to allow it to act. For example, extracts of neutrophils, lymphocytes or platelets may render resistant bacteria susceptible[222], and antibody with complement may penetrate the coating of E. coli to make way for lysozyme[220]. The bacteriolytic ability of lysozyme is used to assay its activity, and lysis of micrococcus lysodeikticus is the technique which is most commonly employed[223-226]. However, immunohistochemical techniques are extremely useful for localising the enzyme[227], and if adequate controls are used, these methods provide a powerful and sensitive research tool. It must be remembered, however, that lysozymes from different species are immunologically distinct!

A major source of lysozyme is the macrophage. The enzyme has been demonstrated in monocytes and macrophages

using bioassay[228], histochemical[229] and immunohistochemical[227] techniques, large amounts of lysozyme accumulate in the serum and urine of patients with monocytic leukaemia[230], and ^{14}C-labelling studies have shown conclusively that monocytes and macrophages from humans and mice synthesise the enzyme[223,231]. Lysozyme is not exclusively confined to macrophages, however, and it has been identified in a wide variety of cells including proximal renal tubular cells, acinar cells in lacrimal and salivary glands, paneth cells in the intestine, serous bronchial cells, and polymorphonuclear leukocytes[227]. The polymorph is the most consistently lysozyme-containing cell of non-macrophage origin[227,232], but it does not appear to be capable of synthesising the enzyme[231], and therefore, although other cells may be capable of accumulating and utilising it, the macrophage may be the only actual source of lysozyme, certainly among blood cells.

Having said this, it is important to stress that not all macrophages have a similar lysozyme content. In studies of rabbit alveolar and peritoneal macrophages, the alveolar cells have been found to contain much higher levels of lysozyme [228,233], indicating either a true heterogeneity, or a variation in the level of activation. Certainly, there is good evidence that lysozyme levels may reflect activation in macrophages. Treatment of mice with macrophage-activating agents such as BCG, zymosan or bacterial endotoxin increases lysozyme activity in spleen[220,234], BCG-induced granulomas produce large amounts of lysozyme[235], and macrophages stimulated by BCG[233] or thioglycollate[223] respond with raised levels of lysozyme.

In rats, intravenous glucan produces both increased serum lysozyme levels and an increased rate of colloidal carbon clearance, and subsequent admininstration of methyl palmitate, which is known to interfere with phagocytic function, causes both of these parameters to return to normal despite the persistance of glucan-induced hepatosplenomegaly[236]. These pieces of evidence would suggest that activated macrophages have elevated levels of lysozyme, and this is supported by immunohistochemical studies which show that macrophages within lymph nodes and other tissues tend to contain detectable lysozyme only when chronic inflammation is present[227,237].

In view of these findings, it is conceivable that lysozyme may provide a measure of macrophage activity in tumour bearing hosts. Serum lysozyme levels are certainly elevated in animals[225,238,239] and in humans[224,226,240] with malignant disease, although unfortunately, this is not tumour specific, as patients with granulomatous disease such as tuberculosis[241], sarcoidosis[242] and Crohn's disease[243] display similar levels. In animals, lysozyme levels appear to rise with progressive tumour growth[225], but in humans, the situation is less straightforward; some workers find that levels tend to be lower in advanced disease[240], but others find virtually the reverse[226].

The origin of lysozyme in the tumour-bearing subject is not clear, although separation studies suggest that tumour cells do not produce significant amounts[225,239]. The work of Currie would indicate that tumour-associated macrophages constitute a major source, since adherent trypsin

resistant cells from rat sarcomata were found to release 7.0 µg of lysozyme per 10^6 cells per day in culture, and similar cells from tumour-draining lymph nodes also produced substantial quantities[225]. However, it should be mentioned that the highest levels of lysozyme came from the macrophages associated with immunogenic, non-metastasising tumours, and to draw a parallel with naturally-occurring human tumours might be misleading. Nevertheless, Currie has shown that adherent cells from human melanomas can produce lysozyme in culture[240], but a further proviso must be made to the effect that lysozyme production has only been demonstrated in cells which have been separated from the tumour environment. It remains to be proved that tumour-infiltrating macrophages can produce lysozyme in situ, and therefore account for the raised serum levels.

Finally, it is of interest to determine whether lysozyme can act as an effector mechanism in tumour cell destruction. Although the bacterial cell wall is the main defined substrate, there is evidence that mammalian cells might also be susceptible. It has been suggested that a great lysozyme load is responsible for damage to the proximal renal tubules in certain leukaemias[230], and lysozyme can cause visible flocculation of liver cell suspensions which on electron microscopy appears to be due to mitochondrial clumping[244]. The enzyme has also been shown to cause the release of hexosamine from acid-insoluble components of chick fibroblasts and Hela cells[245], and profound changes in the surface structure of embryonic fibroblasts and transformed liver

cells has been seen after incubation with lysozyme[246]. Administration of macrophage-activating substances which have anti-tumour effects in mice is accompanied by an increase in serum lysozyme[70,247], and in a study of BCG therapy in human malignant melanoma, patients who responded exhibited a significant rise in serum lysozyme levels[248].

Thus it would appear that lysozyme might have a role to play in anti-tumour immunity, although what that role might be has yet to be clarified. A direct effect on the tumour cell membrane would seem likely, but lysozyme can have an enhancing effect on phagocytosis[249], and may also operate by stimulating macrophage-mediated tumour cell killing[250]. It is therefore possible that the enzyme might act as a positive feedback mechanism, stimulating rather than effecting the tumouricidal abilities of macrophages.

iii) Alpha-1-antitrypsin

Alpha-1-antitrypsin was discovered in 1955 by Schultze and colleagues[251] as alpha-3,5 glycoprotein, and its present name was suggested in 1962[252]. Despite this name, its activity is by no means restricted to trypsin - indeed it is a major anti-protease displaying activity against a wide range of enzymes[253].

Of particular interest in the present context is the fact that alpha-1-antitrypsin is found in monocytes and

macrophages. This was first shown in human pulmonary alveolar macrophages by direct immunofluorescence[254], and it has been demonstrated in tissue macrophages and blood monocytes[255]. Originally, it was thought that monocytes might acquire alpha-1-antitrypsin by uptake from the plasma, but the fact that a human histiocytic cell line contained the substance after three years in culture suggested that synthesis might take place in mononuclear phagocytes[256]. This was confirmed by isoelectric focussing studies of ^{3}H-labelled lysates of short-term cultures which showed that, in human blood monocytes, ^{3}H-leucine was incorporated into material with the immunological and focussing characteristics of alpha-1-antitrypsin[255]. In addition, workers have observed isotopically-labelled alpha-1-antitrypsin in the supernatants of short term cultures of adherent blood monocytes[257]. As with lysozyme, a degree of heterogeneity exists, and only about 50% of human monocytes contain alpha-1-antitrypsin[255], but whether its presence denotes heightened macrophage activity or activation has yet to be established.

The physiological function of alpha-1-antitrypsin is also essentially unknown, but some theories are tenable. Macrophages are known to release various enzymes during phagocytosis[214,215,258,259], and it is thought that this may be due to "spillage" of lysosomal content[260]. If macrophages were capable of neutralising these leaked enzymes, this would constitute an important protective mechanism against proteolysis, but whether alpha-1-antitrypsin is involved in such a process is not yet known.

Further light is shed on alpha-1-antitrypsin by studying patients who are deficient in the substance. Alpha-1-antitrypsin deficiency is genetically determined, following an autosomal dominant pattern, and isofocussing studies have shown that 20-30% of individuals in many populations are not of the normal (MM) phenotype[261]. There is a strong association between the deficiency state and two conditions - liver dysfunction sometimes amounting to childhood cirrhosis[262,263], and pulmonary emphysema[264,265]. A direct causal relationship between alpha-1-antitrypsin deficiency and the hepatic problem has not been established, but in emphysema such a relationship may well exist. Elastase is the main proteolytic component in the production of experimental emphysema, and elastase activity is well recognised in pulmonary alveolar macrophages[265]. It is thought that in alpha-1-antitrypsin deficiency, the major protective mechanism against elastase is absent, and the unopposed effect of this enzyme causes emphysema. Smoking may induce emphysema in a similar manner, as pulmonary macrophages from smokers have elevated levels of elastase, and subjects who are heterozygous for alpha-1-antitrypsin deficiency are highly susceptible to the emphysema-inducing effects of smoking[265]. The source of alpha-1-antitrypsin is not clear, but it may be the pulmonary alveolar macrophages themselves, as in smokers, these cells have elevated levels[266,267], suggesting modulation of an internal protective mechanism.

Alpha-1-antitrypsin may also have a role in immunoregulation. In patients with alpha-1-antitrypsin deficiency, there is a serum-mediated enhancement of lymphocyte

responses to phytohaemagglutinin, accelerated delayed hypersensitivity, and elevated levels of C3 and C5[268]. It has also been shown that alpha-1-antitrypsin can inhibit the phytohaemagglutinin response of human lymphocytes in serum-free medium[269], and partially prevent the adherence and ingestion of C3-coated particles by human monocytes[270]. It is also known that proteases may be mitogenic for lymphocytes[271], and alpha-1-antitrypsin may also modify this process.

The role of alpha-1-antitrypsin in tumour biology is even less well understood. Increased serum levels are seen in many patients with malignant disease[272-274], but this is decidedly non-specific as it is also seen in burns[275], pregnancy[276], and in the response to typhoid vaccine[277]. Alpha-1-antitrypsin does seem to be present in some tumours, however, and using immunohistochemical methods, it has been detected in hepatomas[278] and ovarian carcinomas[279], both in the stroma and in cells. Whether it originates from tumour cells or host cells is unknown, and whether it has any anti-tumour effect is equally obscure, but there is a suggestion that the incidence of malignancy might be increased in patients with alpha-1-antitrypsin deficiency. Patients with hepatoma, myeloma or lymphoma may be more likely to be heterozygous than comparable controls[280,281], and there has also been a report from Finland of an increased frequency of heterozygotes among patients with squamous carcinoma[282].

It has been suggested that alpha-1-antitrypsin might act as an inhibitor of cell division in the control of hepatic regeneration[283], and this may have implications for

tumour growth. However, it is also possible that the presence of alpha-1-antitrypsin within a tumour may protect it from the effects of host-derived proteolytic enzymes. Clearly, research into alpha-1-antitrypsin has a long way to go before its role, if any, in the mediation of tumour behaviour can be defined.

Discussion and Conclusions from the Literature

Although there is convincing evidence that most tumours contain macrophages (q.v. pages 6 - 7) the role of these cells in the biology of cancer is still obscure. Much of the vast body of research pertaining to this subject has been done using animal systems, firstly because animal tissue is more readily available than human tissue, and secondly because extensive in vivo manipulations are possible. However, experimental tumour models are legion; consequently, the behaviour of one system is not necessarily mirrored by another, and in some cases quite contradictory results can be obtained from similar experiments on tumours of different origins and aetiologies. This is obviously a grave disadvantage, especially when one is tempted to make generalisations and to extrapolate them to naturally-occurring human neoplasms. Similarly, comparisons between different models is not necessarily a useful exercise; animal tumour systems are often homogeneous in terms of their natural history, and differences in macrophages associated with tumours of differing behaviour are often interpreted as causal phenomena. The information provided by such studies may not be relevant to human tumours, because a naturally occurring neoplasm usually displays a wide spectrum of growth and dissemination patterns, and therefore interest tends to focus on the ability of the macrophage to modify the behaviour of a single tumour type.

Despite these problems, it is possible to draw some general conclusions from the literature. There can be

no doubt that some macrophages can kill some tumour cells in vitro by a variety of mechanisms (q.v. pages 8 - 14). The main questions to ask, therefore, are

a) Do macrophages have any anti-tumour effects in vivo?

b) Why are macrophages unable to destroy established tumours in their natural surroundings?

Studies with anti-macrophage agents suggest that macrophages may well be exerting a restraining influence on tumours (q.v. page 10), but the association between the administration of these agents and tumour progression is not absolute proof of this. Similarly, the relationship between macrophage content and spontaneous or induced tumour regression (q.v. page 11) does not necessarily cast the macrophage in the role of causal agent. Unfortunately, therefore, incontrovertible evidence that macrophages are actively combating tumours in the in vivo situation is still lacking.

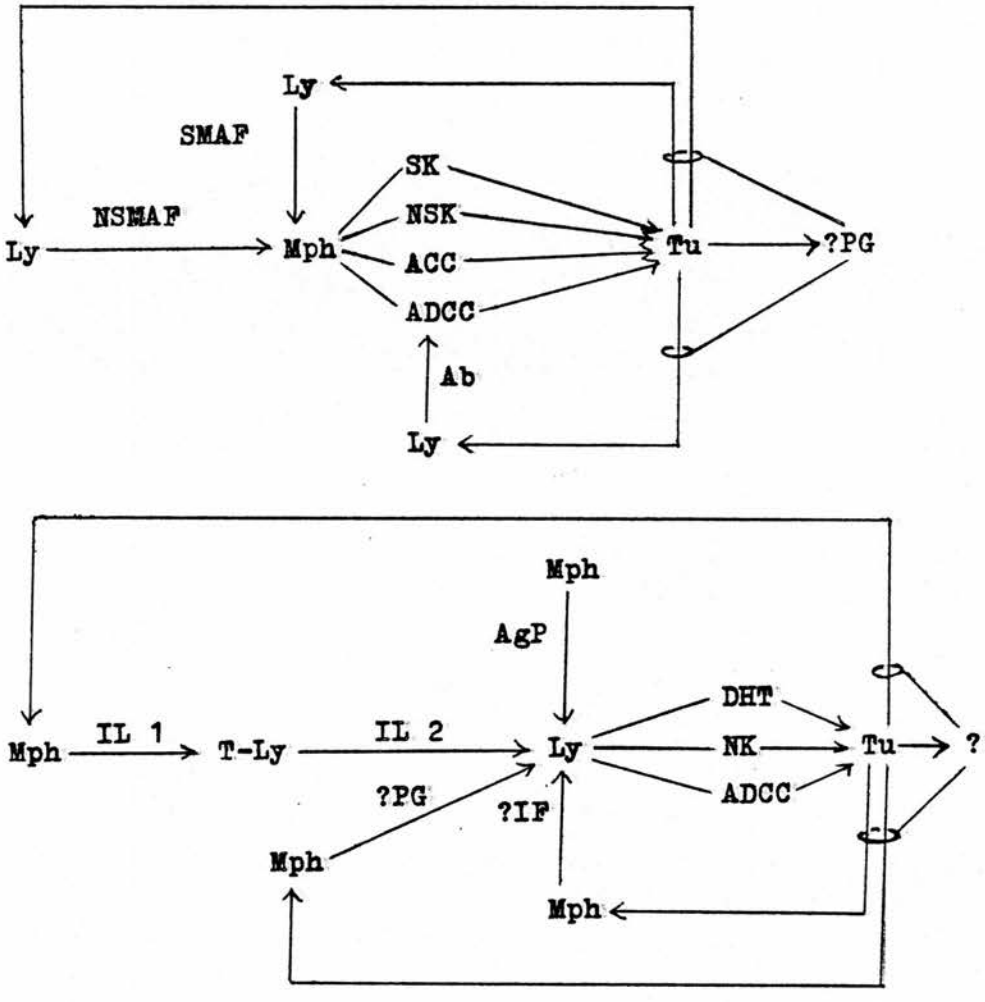
Turning to the second question, the fact that macrophages can kill tumour cells in vitro, but do not appear to have a significant effect under normal in vivo conditions, leads to the conclusion that tumours may be capable of subverting macrophage function. In some respects, however, tumours appear to be stimulatory, and macrophage proliferation is a common finding (q.v. pages 17 - 18). Presumably this represents a form of host response to the neoplasm, as does accumulation of macrophages at the tumour site (q.v. pages 6 - 7), but most tumour effects on macrophages appear to counter effective eradication of the neoplastic cells. In particular,

chemotaxis, phagocytosis and Fc receptor expression can be depressed by tumour factors (q.v. pages 27 - 28) and it is possible that host macrophages may be modified by the tumour so that they actually aid tumour growth (q.v. pages 14 - 15). Paradoxically, cytotoxic macrophages can be isolated from tumours (q.v. pages 12 - 13), but their tumouricidal ability can only be demonstrated after removal from the neoplastic environment, and the activation state of in-situ tumour-infiltrating macrophages has yet to be established.

The research detailed in this thesis is not directly concerned with lymphocyte-macrophage interactions, but such collaberation is so central to the function of mononuclear phagocytes that no discussion of the macrophage in cancer is complete without at least a limited discussion of the role of the lymphocyte. Pages 20 - 25 contain the details, but essentially two possible situations may exist or even co-exist. Firstly, the direct effect of macrophages on tumour cells may be triggered or modulated by lymphocytes, and, in turn, the effect of tumours on macrophages may be mediated by lymphocytes. Secondly, the effect of macrophages on tumours may be mediated by lymphocytes, and conversely, the tumour may abrogate lymphocyte effects via macrophages, thus giving the impression that the macrophages are forming a "fifth column". The mechanisms whereby these interactions may take place are outlined in Figure 1, although it should be stressed that they are not all firmly implicated in anti-tumour host responses.

Among the measurable properties of tumour-associated

Figure 1



Mph	-	Macrophage
Ly	-	Lymphocyte
Tu	-	Tumour

Ab	-	Antibody
Acc	-	Accumulation
ADCC	-	Antibody-dependant cytotoxicity
AgP	-	Antigen presentation
DTH	-	Delayed-type hypersensitivity
IF	-	Interferon
IK	-	Interleukin
NK	-	Natural killing
NSK	-	Non-specific killing
NSMAF	-	Non-specific macrophage activating factor
PG	-	Prostaglandin
SMAF	-	Specific macrophage arming factor

macrophages, surface characteristics have been particularly extensively studied, and are of importance for two reasons. Primarily, they can be used to identify macrophages in tumour cell suspensions, where morphological examination is insufficient, and for this purpose, the Fc (IgG) receptor has proved a useful tool although not without theoretical disadvantages (q.v. pages 6 - 7). Surface receptors may also provide a means of assessing cell activation, however, (q.v. pages 28 - 29) and using the parameter of Fc (IgG) receptor expression, it has been suggested that blood monocytes from cancer patients are activated whereas tumour-infiltrating macrophages are not (q.v. pages 27 - 28). This phenomenon requires further study, and other surface characteristics such as C3 receptors and Ia antigens are worthy of more detailed study in patients with cancer.

Phagocytosis is another property of macrophages which is subject to alteration by tumours. In general, this parameter appears to be depressed in tumour-bearing animals (q.v. pages 34 - 35), but the evidence for this is not particularly strong, and comparative studies of the phagocytic capacity of tumour-infiltrating macrophages need to be done. It would seem, however, that phagocytosis is unlikely to be an effector mechanism in tumour cell killing (q.v. page 35) although it may be a useful measure of macrophage activation.

Finally, cytoplasmic factors may be important in macrophage-mediated anti-tumour reactions, and in this thesis, two have been selected for study - lysozyme and alpha-1-anti-trypsin. Lysozyme is synthesised by macrophages, and there

is good evidence that lysozyme levels in these cells provide a measure of activation (q.v. pages 39 - 40). Further interest in lysozyme is generated by the finding that it can damage tumour cells (q.v. pages 41 - 42) and the ability of macrophages to produce the enzyme may be an important factor in anti-tumour mechanisms. Tumour-bearing hosts frequently have raised serum lysozyme levels, but although some workers have suggested that this originates from tumour-infiltrating cells, evidence for the actual production of the enzyme by in-situ tumour-associated macrophages is lacking, and further research is indicated in this sphere.

Alpha-1-antitrypsin is a major protease inhibitor in macrophages which is probably synthesised by them, and which may play an important role in the regulation of lysosomal enzymes discharged from the cell (q.v. pages 43 - 44). Its place in the host response to tumours is unknown, although there has been speculation that alpha-1-antitrypsin deficiency may predispose to tumour formation (q.v. pages 45 - 46), and it would be of great interest to know whether macrophages associated with tumours display any alterations in this substance.

From the literature review it is clear that many unresolved problems remain, and macrophages associated with spontaneous human tumours have been very little studied. In the experimental work described in this thesis, human breast cancer has been studied, and it was elected to concentrate on blood monocytes, macrophages from regional lymph nodes, and tumour-infiltrating macrophages. The properties studied include expression of Fc receptors, C3 receptors and Ia

antigens, phagocytosis of IgG-coated erythrocytes, and lysozyme/alpha-1-antitrypsin content. These markers have been used to characterise the tumour associated macrophages and to compare them with normal cells. In order to introduce the original studies, seven questions are now posed, in an order corresponding to that in which the relevant studies are subsequently documented.

i) Can tumour cells display the surface markers used to identify macrophages?

ii) Is the lysozyme content of macrophages altered by the presence of tumour?

iii) Is the alpha-1-antitrypsin content of macrophages altered by the presence of tumour?

iv) Do tumour-associated macrophages display altered expression of Fc receptors, C3 receptors or Ia antigens?

v) Do tumour-associated macrophages have altered phagocytic ability?

vi) What is the nature of sinus histiocytosis in regional lymph nodes draining tumours?

vii) Does the proportion of a tumour made up of macrophages correlate with prognosis?

The following section on original work describes how an attempt has been made to answer these questions. In addition, some observations on the function of normal macrophages have been made, and these are also described.

ORIGINAL WORK

1. Materials and General Methods

i. List of abbreviations

Unless commonly used, abbreviations have not been employed without an explanation in the text or in the appropriate footnote or legend. However, for convenience, a full list of all abbreviations is given below.

A1AT	-	Alpha-1-antitrypsin
AD	-	Distilled water
BPA	-	Bovine plasma albumin
BT2/9	-	Code name for anti-Ia antigen mouse monoclonal antibody
C3	-	Third component of complement
EA	-	Erythrocyte-antibody (refers to indicator used for detecting Fc (IgG) receptor-bearing cells)
EAC	-	Erythrocyte-antibody-complement (refers to indicator used for detecting C3 receptor-bearing cells)
EMA	-	Epithelial membrane antigen
Fc	-	The Fc fragment of immunoglobulin G
FCS	-	Fetal calf serum
g	-	Unit gravity
Ia	-	Immune response associated
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
Mϕ	-	Macrophage/monocyte
My6C3	-	Code name for mouse monoclonal antibody reacting with macrophage/monocyte associated antigen

NSS - Normal swine serum

P - Probability value

PAP - Peroxidase-anti-peroxidase conjugate

PBS - Phosphate buffered saline

rpm - Revolutions per minute

SAR - Swine anti-rabbit immunoglobulin

1 ii. Patients

The patients studied were all under the care of Professor A.P.M. Forrest, Department of Clinical Surgery, University of Edinburgh, and all gave informed consent, not only to have appropriate surgery carried out, but also to donate the extra blood samples required for the study. The cancer patients all had invasive breast cancer (confirmed histologically) which was clinically confined to the breast and axillary lymph nodes, and blood samples were taken immediately before premedication prior to surgery. The diagnosis of carcinoma had been established pre-operatively by fine needle aspiration cytology, and none of the patients studied had undergone "tru-cut" or open breast biopsy.

The control patients had a wide variety of non-neoplastic, non-inflammatory surgical conditions, and they too had blood samples taken immediately before sedation preceding surgery, to try to eliminate any effect that pre-operative stress might have on the parameters to be measured.

The pathological details of both the cancer patients and the control patients are given in the appendix. For the cancer patients, four prognostic factors were recorded:

1. Lymph node status - whether or not the axillary lymph nodes were invaded by tumour
2. Tumour size - maximum diameter of the tumour, measured on the resection specimen
3. Tumour grade - histological tumour grade, assessed by the method of Bloom and Richardson

4. Oestrogen receptor status - estimated by standard saturation analysis

Details of the methods of assessing these four factors are given in Part 1 of this thesis (Volume 1, pages 115 - 124).

1 iii. Preparation of blood mononuclear cells (Figure 2)

The patients were bled from an antecubital vein into plastic syringes containing enough preservative-free heparin[i] to obtain a concentration of 10 units per ml. of blood. The blood was then diluted with phosphate-buffered saline (PBS)[ii] at a ratio of 1(blood): 2(PBS), and 10 ml. aliquots of this mixture were layered onto Ficoll-Hypaque[iii] in 50 ml. siliconed glass tubes. After the tubes had been carefully balanced, they were spun with an interface force of 400g for 40 minutes at 20°C in a "Mistral 6L" centrifuge. The interface layer, which contained the mononuclear cells, was then removed by means of a glass pipette, and washed twice in medium[iv] with 2% fetal calf serum (FCS)[v] by spinning the cells down in the medium at 400g for 15 minutes. After the second wash, the cells were counted and their viability assessed using a Neubauer chamber and phase contrast microscopy. The cells were finally resuspended in medium containing 10% FCS at 2×10^6 per ml.

i. Heparin - Heparin sodium B.P. (mucous) with no bactericide. 1000 i.u. per ml. PAINES AND BYRNE Ltd.

ii. PBS -

NaCl	8000 mg
KCL	200 mg
Na_2HPO_4	1150 mg
KH_2PO_4	200 mg
	A.D. to 1000 mls.

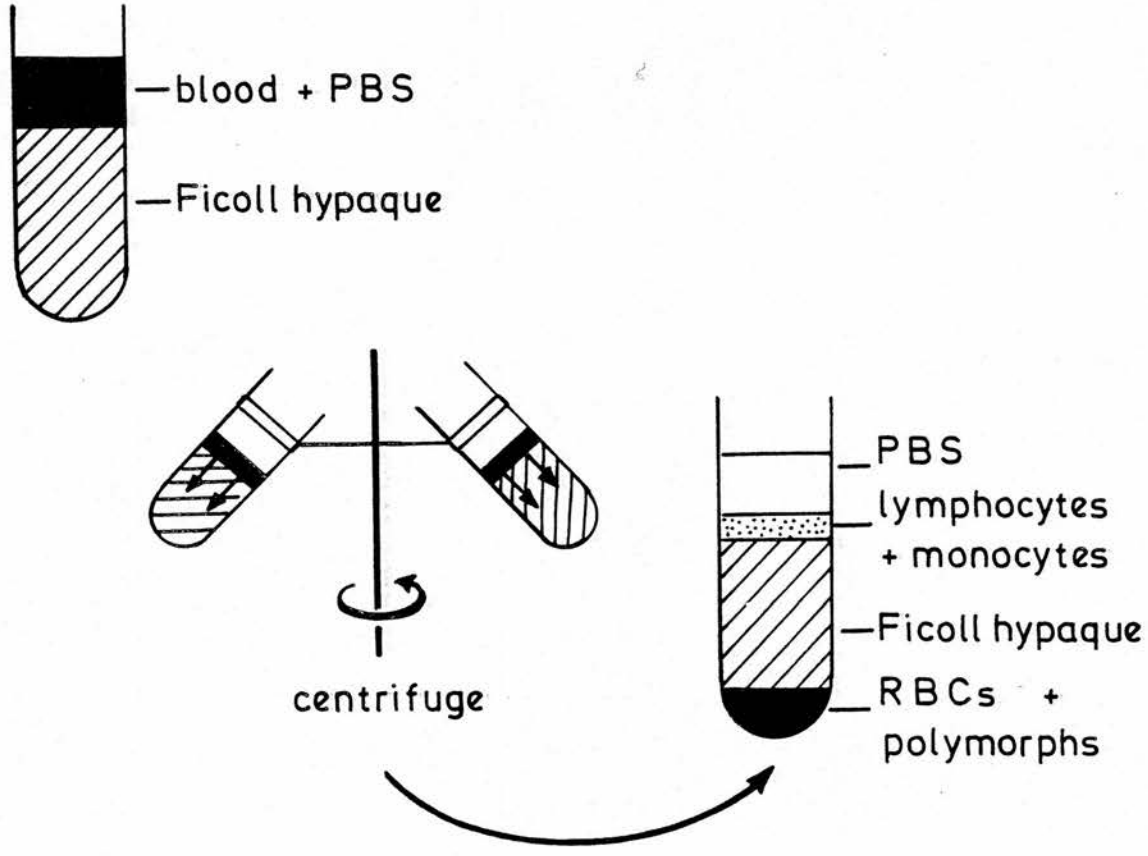
iii. Ficoll-Hypaque - Ficoll 4000, PHARMACIA
Hypaque sodium (sodium diatrizoate B.P.), STERLING RESEARCH LABORATORIES.

Ficoll 63.5 g
Hypaque 100 g
Made up to 1 litre with A.D. Density 1.0770 g/ml (checked with density bottle)

iv. Medium - RPMI 1640, GIBCO
plus 0.1 g/l streptomycin sulphate B.P., GLAXO
plus 100,000 u/l benzyl penicillin (sodium) B.P., GLAXO
plus 0.7 g/l NaHCO3
plus 25 mM Hepes buffer (N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid), HOPKINS AND WILLIAMS

v. FCS - GIBCO. All batches 0.1μ filtered to assure freedom from mycoplasma. Heat inactivated at 56°C for 45 minutes before use.

Figure 2 Preparation of blood mononuclear cells by separation on a Ficoll-Hypaque gradient.



1 iv. Preparation of cells from mammary carcinomas (Figure 3)

After removal of the tumour from the breast, a portion was taken for histology, and the remainder placed directly into medium containing 10% FCS. The tumour was then transferred to a microbiological safety cabinet[i] to avoid bacterial contamination, and after it had been trimmed of fat and non-tumour tissue it was disaggregated as thoroughly as possible using a scalpel blade (size 16). The tumour fragments were then placed in collagenase[ii], and incubated for 12 hours at 37°C. After incubation, the container was shaken vigorously and the contents filtered through muslin gauze to separate the cells from the larger residual pieces of stroma and debris. The cells were then washed six times in medium containing 2% FCS at 400g in an "MSE Minor" bench centrifuge, and then resuspended in medium containing 10% FCS. The cells were counted and their viability assessed using the Neubauer chamber and phase contrast microscopy, and finally resuspended in medium containing 10% FCS at 2×10^6 per ml.

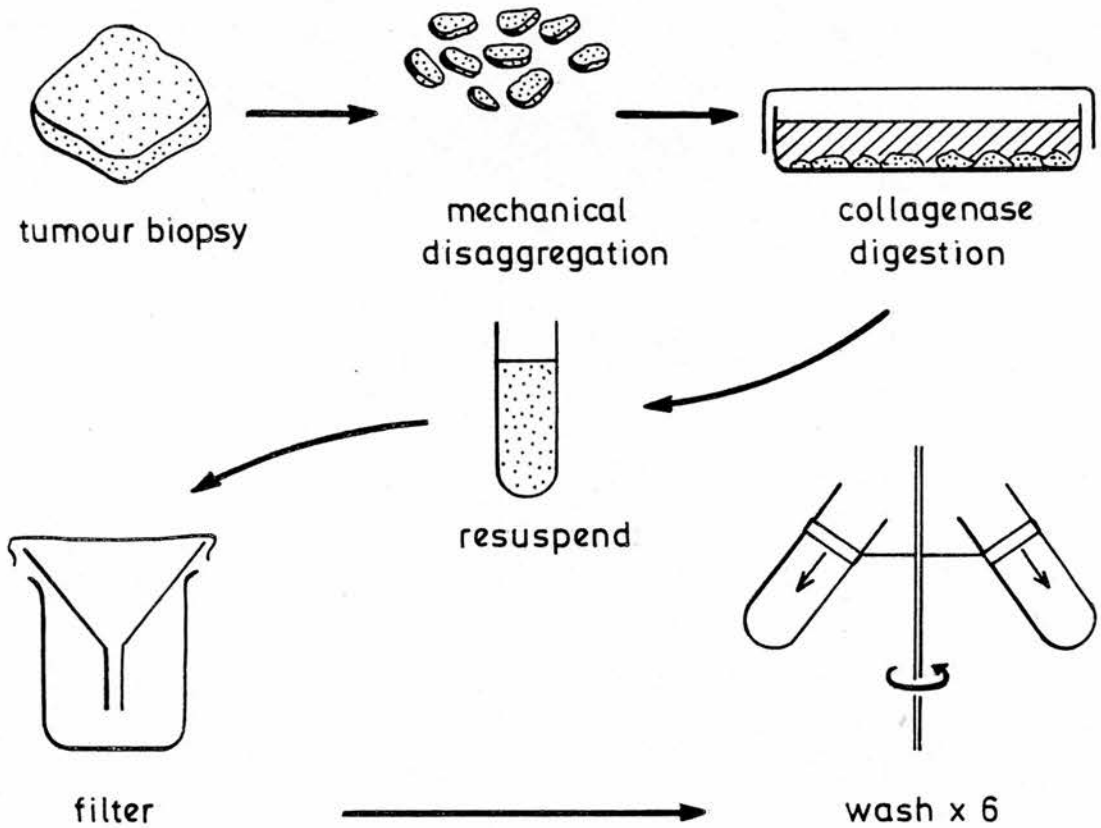
i. Safety cabinet - Envair unidirectional laminar downflow microbiological safety cabinet

ii. Collagenase - Type IA, derived from clostridium histolyticum, SIGMA.

Collagenase	300	units/mg dry wt.
Clostripain	0.94	units/mg dry wt.
N/S protease	232	units/mg dry wt.
Trypsin	0.09	units/mg dry wt.

This is made up in medium (see page 61) to 300 units collagenase (i.e. 1 mg) per ml, filter sterilised and stored at -20°C

Figure 3 Preparation of tumour.



1 v. Preparation of mononuclear cells from lymph nodes

After removal of the lymph node from the axillary fat, it was placed directly into medium containing 10% FCS. It was then transferred to the safety cabinet, and a section taken for histology. Multiple puncture with a fine hypodermic needle was then performed, and lymphocytes expelled from the node into the surrounding medium through the needle. The remaining stroma was then mechanically disaggregated using a scalpel, and incubated in collagenase for 12 hours at 37°C, to isolate the retained macrophage-like cells (see note). After incubation, the container was shaken vigorously, and the contents filtered through muslin gauze to separate cells from any remaining fragments of stroma. The cells were then washed six times in medium containing 2% FCS at 400g in an "MSE Minor" bench centrifuge, and then resuspended in 20 mls. of medium with 10% FCS. The cells were counted and their viability assessed using the Neubauer chamber and phase contrast microscopy, and finally resuspended in medium containing 10% FCS at 2×10^6 per ml.

Note: The use of collagenase was found to be necessary because if multiple puncture and mechanical disaggregation alone were used very few of the cells obtained were macrophage-like, most of them being lymphocytes. Histological examination of residual stroma after mechanical extraction of cells showed there to be many histiocytic cells adhering to the strands of fibrous tissue. Collagenase treatment of the residual stroma yielded a much higher percentage of macrophage-like cells, as determined by phase contrast microscopy and on cyto-centrifuge preparations, than did mechanical extraction.

1 vi. Preparation of indicator erythrocytes

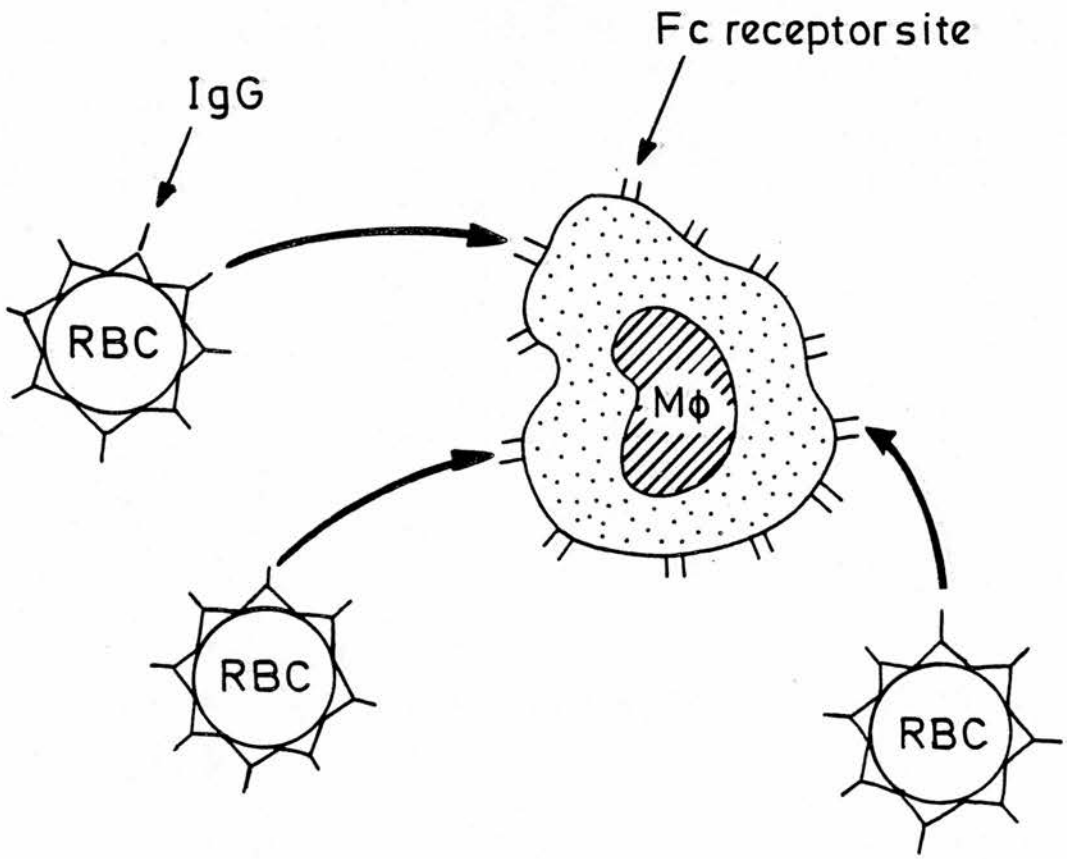
a) Fc receptor indicators

The purpose of these cells is to indicate the presence of receptors for the Fc portion of IgG on the surface of macrophages by rosette formation (Figure 4). Heparinised whole ox blood[i] was washed four times in PBS by spinning at 1000g for 5 minutes in an "MSE Super Minor" bench centrifuge, and the packed red blood cells were then resuspended at 2% in PBS. To make 1 ml. of Fc receptor indicator, 0.5 mls. of 1:100 rabbit IgG anti-ox erythrocyte antibody[ii] in PBS was added to 0.5 mls. of the 2% suspension of ox erythrocytes in PBS. This mixture was incubated at room temperature for 45 minutes, washed twice in PBS at 400g for 5 minutes and resuspended in 1 ml. of medium with 10% FCS. Once prepared, the indicators were stored at 4°C and could be used over a seven day period.

Note: It is usual to refer to Fc receptor detecting rosettes as EA (erythrocyte-antibody) rosettes

i.	Ox blood	-	Gift from Professor R.R.A. Coombs, Department of Immunology, University of Cambridge
ii.	Rabbit IgG anti-ox erythrocyte antibody	-	Gift from Professor R.R.A. Coombs, Department of Immunology, University of Cambridge

Figure 4 Formation of erythrocyte antibody rosettes to detect Fc receptors.



b) C3 receptor indicators

The purpose of these cells is to indicate the presence of C3 receptors on the surface of macrophages by rosette formation. Heparinised whole ox blood was washed four times in PBS by spinning at 1000g for 5 minutes in an "MSE Super Minor" bench centrifuge, and the packed red blood cells were then resuspended at 2% in PBS. To make 1 ml. C3 receptor indicator, 0.5 ml. of 1:40 rabbit IgM anti-ox erythrocyte antibody in PBS was added to 0.5 ml. of the 2% suspension of ox erythrocytes. This mixture was incubated for 15 minutes at 4°C, washed twice in PBS at 400g for 5 minutes, and resuspended in 0.5 mls. of complement fixation test diluent (CFTD)[ii]. To this suspension was added 0.5 mls. of 1:10 C5-deficient mouse serum[iii] in CFTD, and the resultant mixture was incubated at 37°C for 15 minutes. The cells were then washed twice in PBS at 400g, and resuspended in 1 ml. of medium containing 10% FCS. Ox erythrocytes coated with IgM in the absence of C3 were used as controls.

Note: It is usual to refer to C3-detecting rosettes as EAC (erythrocyte-antibody-complement) rosettes

i.	Rabbit IgM anti-ox erythrocyte antibody	-	Gift from Professor R.R.A. Coombs, Department of Immunology, University of Cambridge
ii.	CFTD	-	OXOID. Made up by adding 1 tablet to 100 mls. A.D.
iii.	C5-deficient mouse serum	-	Gift from Professor R.R.A. Coombs, Department of Immunology, University of Cambridge. Obtained from AJ mice; mice bled and blood allowed to clot at room temp. Serum removed, and absorbed with washed and packed ox erythr. Resultant C5-deficient serum stored at -180°C

c) Indicators for the Direct Antiglobulin Rosetting Reaction

The Direct Antiglobulin Rosetting Reaction provided a means whereby specific antibody molecules were coupled to the surface of erythrocytes so that when these coated cells were brought into contact with cells under test (in this case, macrophages), rosette formation identified cells bearing target antigen (Figure 5). This assay is derived from the mixed agglutination test of Coombs[284,285], and the agent used for coupling antibody to erythrocytes was chromic chloride[286]. The sensitivity of the test is increased if surface neuraminic acid residues are first stripped off by trypsin treatment of the red blood cells[287], and this technique was also utilised. One of the theoretical disadvantages of this method is the possibility of binding of the indicator cells to the test cells via the Fc receptor. Ling and Richardson, however, report that this does not occur because of the unavailability of the Fc portion of the coupled antibody[285]. Nevertheless, it is wise always to carry out control experiments using appropriate erythrocytes coated with IgG from the same species.

In the present series of experiments, the two antibodies used in this assay were both monoclonal mouse IgG antibodies. One was directed against a surface antigen on human monocytes/macrophages, and was called My6C3[i]; the other was directed against the human Ia antigen, and was called BT2/9[ii]. The method employed for coupling is described below.

Heparinised whole sheep blood[iii] was washed six times in PBS by spinning at 1000g for 5 minutes, and the packed

red blood cells were then resuspended at 10% in PBS. 1 ml. of prewarmed (to 37°C) 10% red blood cell suspension was then mixed with 1 ml. of 0.25% trypsin (pH 7.0)[iv], and this was left for 30 minutes at 37°C in a waterbath. After 30 minutes, the red cells were spun down and washed twice in PBS by spinning at 400g for 5 minutes. Following the wash, the cells were resuspended in 4 mls. of 1:40 1% stock trypsin inhibitor[v] (100 µl.) in PBS, and left for 10 minutes at room temperature. The cells were then spun down and washed twice in 0.9% NaCl by spinning at 400g for 5 minutes.

For the actual coupling procedure, 25 µl. of the immunoglobulin (2 mg of protein/ml.) to be coupled was placed in a tube, and 25 µl. of the packed trypsin-treated red cells were added. To this was slowly added 50 µl. of 0.02% chromic chloride in 0.9% saline, with constant mixing using a vortex mixer; the tube was then capped, and rotated slowly (1 r.p.m.) for 60 minutes at room temperature. At the end of the rotation period, the indicator cells were washed three times in PBS by spinning at 400g for 5 minutes, and resuspended in 2.5 mls. of PBS. To act as controls, red cells coupled to normal mouse immunoglobulin[vi] were prepared in a similar manner. Both the test and control indicator cells were finally washed in PBS and resuspended in PBS containing 1% bovine plasma albumin (BPA)[vii] and stored at 4°C. The same indicators were used, provided no red cell lysis had occurred.

i. My6C3 - Gift from Dr. D. Kraft, Department of Experimental Pathology, University of Vienna

ii. BT2/9 - Gift from Dr. H. Waldman, Department of Immunology, University of Cambridge

iii. Sheep blood - Gift from Professor R.R.A. Coombs, Department of Immunology, Univeristy of Cambridge

iv. Trypsin - 1:250, DIFCO
This was made up in a balanced salt solution as follows:

NaCl	8000 mg
KCL	400 mg
$NaHPO_4$	120 mg
Glucose	1000 mg
Phenol red	10 mg
Trypsin	2500 mg
A.D.	1000 mg

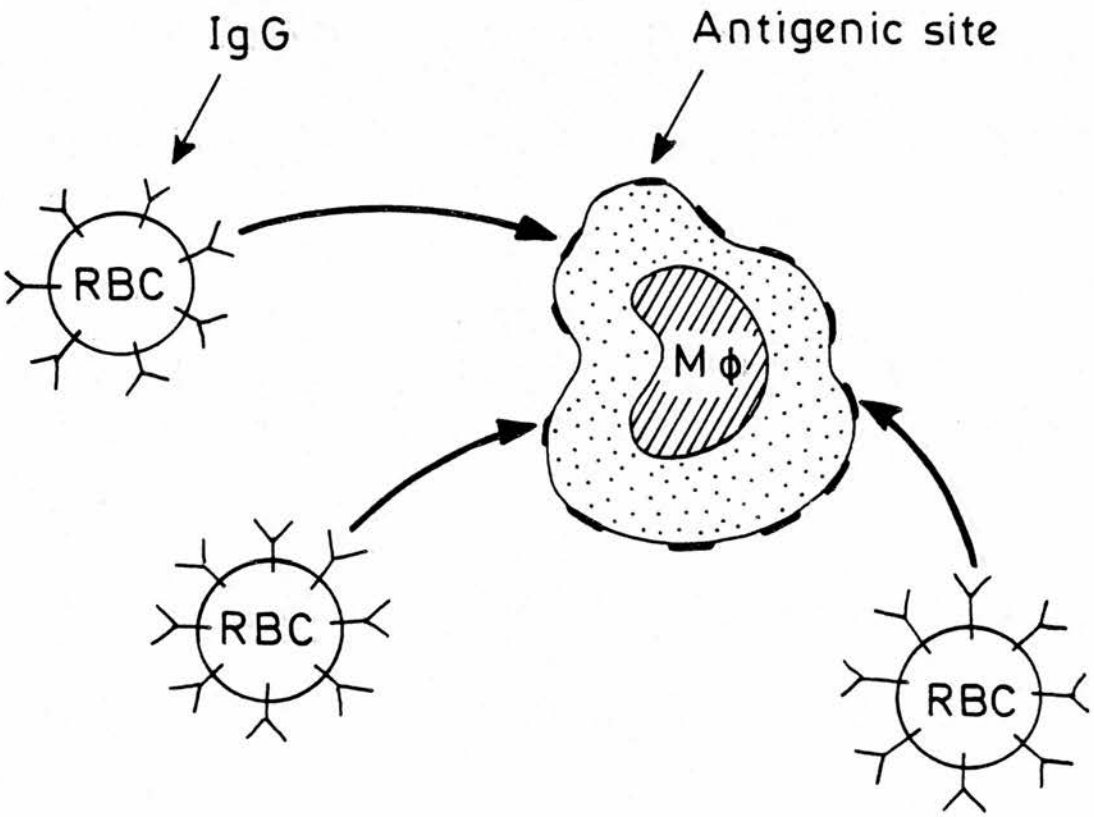
pH was adjusted to 7.4 with 5% $NaHCO_3$ before use

v. Trypsin inhibitor - Type 1-5, SIGMA
Lyophilised from soya bean

vi. Normal mouse immunoglobulin - Mouse gammaglobulins, fraction II. MILES LABORATORIES INC.

vii. BPA - Crystallised bovine plasma albumin ARMOUR PHARMACEUTICAL CO LTD.

Figure 5 The direct antiglobulin rosetting reaction.



d) Agglutination test to check coupling

In order to confirm that coupling of antibody to red cells had taken place, the following procedure was carried out after each coupling had been done. Eleven dilutions of rabbit immunoglobulins against mouse immunoglobulins[i] were made up in PBS, starting at 1:40, and then progressive 1:4 dilutions. One drop of each dilution was then placed into individual wells of a microtitre plate, with 1 drop of PBS in well 12 to check for autoagglutination. One drop of indicator being tested was then placed in each of the wells, and the plate was read after two hours, erythrocyte agglutination indicating that coupling had taken place.

i. Rabbit immunoglobulins against mouse immunoglobulins - DAKO, Z109.

1 vii. Rosette formation

The procedure for producing erythrocyte rosettes using the indicators described in the preceding pages was similar for each indicator, and is outlined below.

Firstly, the cells to be tested were counted in a Neubauer chamber, and enough cells for the various rosette preparations were aliquoted (0.1 ml. of cells at 2 x 10^6/ml. for each preparation) and placed on ice. Fc and C3 receptor indicators were then washed three times at 4°C in medium containing 10% FCS by spinning at 400g for 5 minutes in an "MSE Chilspin" bench centrifuge, and resuspended in medium with 10% FCS at 2 x 10^6 cells/ml. In the case of the coupled indicators, these were washed as above in PBS, and resuspended in PBS with 1% BPA.

Tubes were then labelled, and 100 µl. of appropriate erythrocyte indicator suspension was added to each tube, keeping it on ice at all times. 100 µl. of the test cell suspension was then added to each tube, the mixture was spun at 4°C for 2 minutes at 200g, and the pellet was then incubated, along with the supernatant, at 4°C for 30 minutes. After incubation, the cells were resuspended by gentle rotation for 30 seconds, and, by convention, a resette was taken to comprise a test cell with three or more indicator erythrocytes adhering to it.

1 viii. Cytocentrifuge preparations

The cytocentrifuge used was a "Shandon Cytospin 2" (Figure 6). Essentially, this is a device for depositing cells on to a microscope slide which has a range of operating speeds of 200-2000 r.p.m. After several trials, it was decided to use 800 r.p.m. (approx. 60g) for 5 minutes in order to make preparations of rosettes.

Samples were centrifuged in special plastic sample chambers. Each sample chamber assembly (Figure 7), which comprises sample chamber, filter card and glass microscope slide, was held in position by a special steel slide clip, and up to 12 of these assemblies could fit into the sealed centrifuge head. During centrifugation (at 200 r.p.m.) these assemblies tilted from an angled loading position into an upright operating position. The principle of operation is based on the fact that, because the cell is more dense than the suspending fluid, under an applied force the cell will have greater momentum than the fluid. Thus, after passing through the sample chamber, the cell is projected towards the microscope slide with sufficient momentum to cross the gap formed by placing the filter card between the sample chamber and the slide. The suspending fluid is absorbed into the filter card by capillary action.

In order to produce an optimum concentration of rosettes in the cytocentrifuge preparations, the rosetted cell suspensions were diluted 1:10 in either medium or PBS accordingly. Two drops of this suspension were then placed in each sample chamber, and the resulting preparation was fixed for exactly five minutes in absolute ethanol.

Figure 6 The cytocentrifuge.

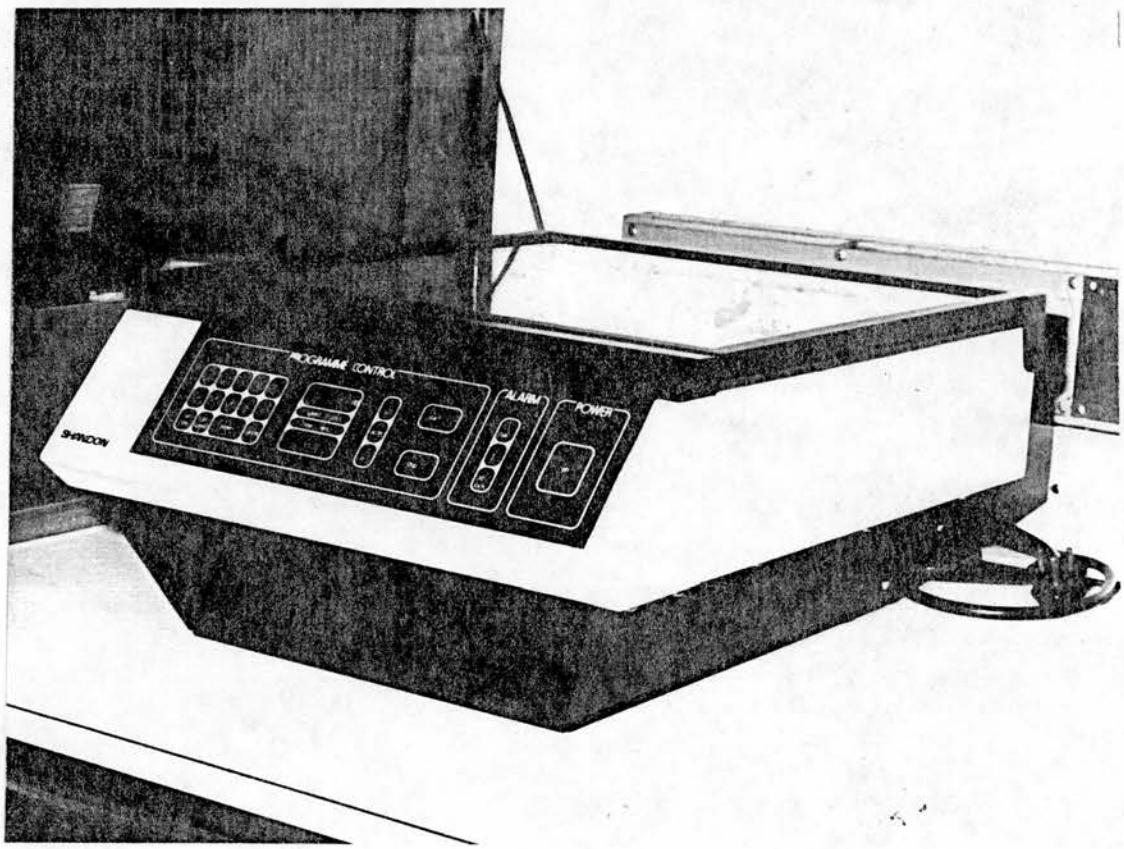
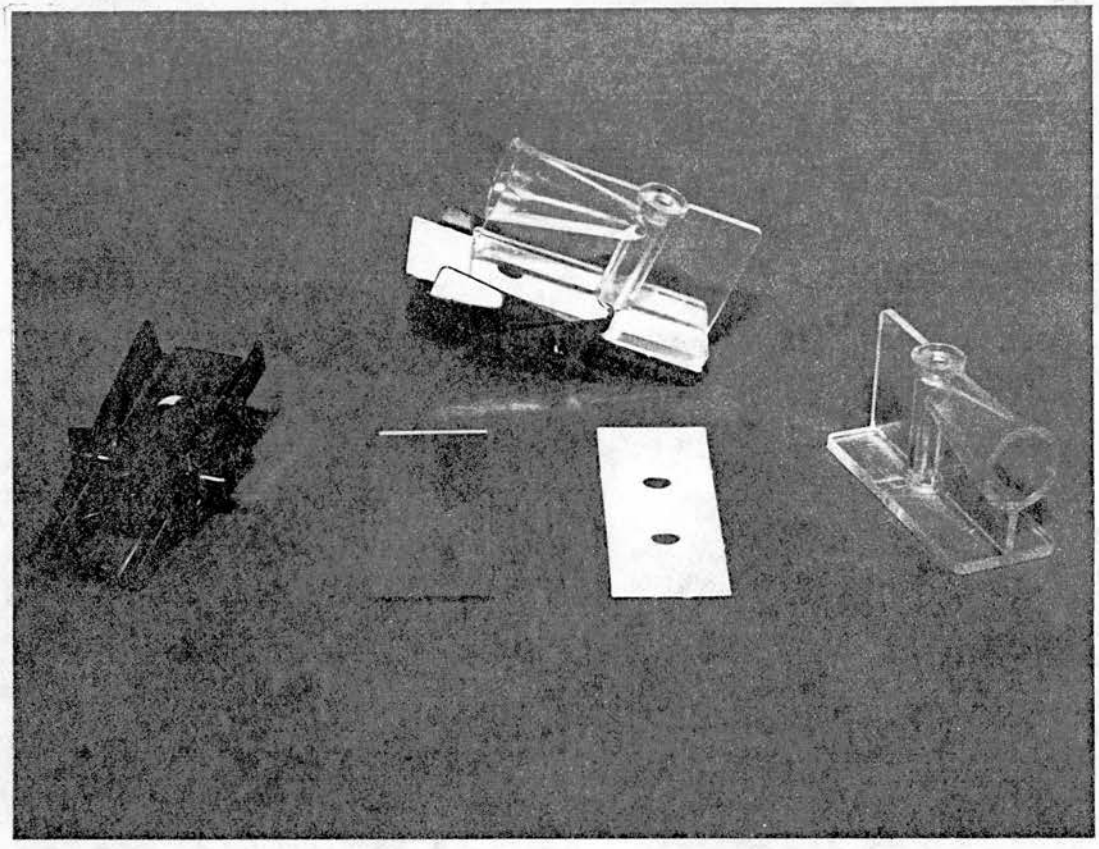


Figure 7 The cytocentrifuge sample chamber assembly.

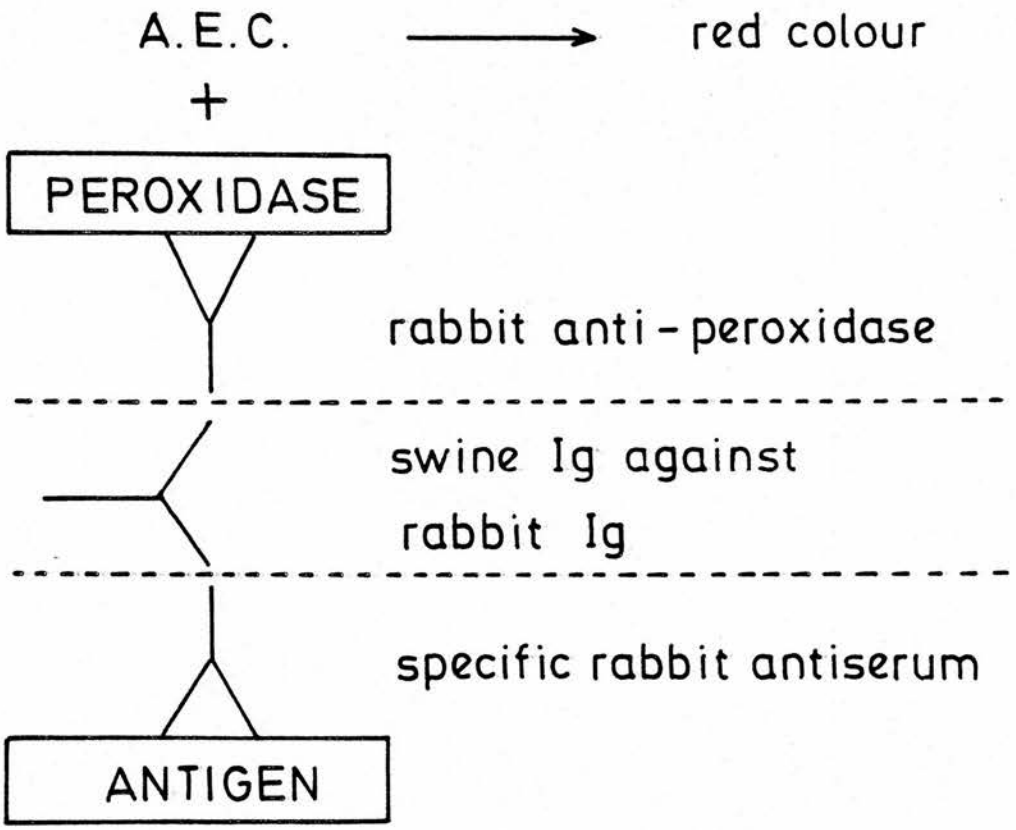


1 ix. Immunoperoxidase technique

The peroxidase-anti-peroxidase (PAP) method of Burns[288] was used for the detection of lysozyme, alpha-1-antitrypsin and epithelial membrane antigen on cytocentrifuge preparations and tissue sections. Essentially, the technique involves incubating antigen-bearing tissue with specific rabbit antibody, followed by swine immunoglobulin against rabbit immunoglobulin, followed by rabbit immunoglobulin against horseradish peroxidase conjugated with peroxidase, and then visualising the peroxidase by means of a substance which forms an insoluble coloured polymer in the presence of the enzyme (Figure 8). Traditionally, diaminobenzidine is used for visualisation[289], but as this is reputed to be carcinogenic, aminoethyl carbazole 0.4% in dimethyl formamide was substituted. This has the disadvantage of being soluble in alcohol, necessitating an aqueous mountant, but otherwise it proved entirely satisfactory, producing a red colour which contrasted well with haematoxylin counterstain.

The PAP method has several advantages over other methods. It is extremely sensitive as the triple layer provides considerable amplification, and because it uses peroxidase, it provides a permanent record and dispenses with the need for fluorescence microscopy. Another important consideration is background staining, and provided the tissue is kept in fluid containing swine serum to block non-specific sites which can otherwise bind the swine anti-rabbit immunoglobulin, this problem is almost completely avoided by using the PAP technique.

Figure 8 The immunoperoxidase assay.



Over-exposure to fixative can impair immunostaining [290-292], presumably by masking antigen. To avoid fixation-associated variations, therefore, all cytospin preparations used for the immunoperoxidase experiments were fixed for exactly 5 minutes in the same fixative (absolute alcohol). Formalin-fixation of tissue sections was not so uniform, however (although the variation was only 1 - 3 days), and to improve immunostaining, trypsin treatment was used for sections in order to "unmask" the antigen[293].

The experimental method and details of the reagents are given in table 1 and the accompanying notes, but two points of technique are worth mentioning. Firstly, the washing of slides in tris-buffered saline, with or without swine serum, was achieved using a magnetic stirring device with the slides suspended in a staining dish (Figure 9). Secondly, when antibody was layered on to the slides (steps 5, 7 and 9), the slides were kept in a transparent wet chamber (foreground, Figure 9) which allowed premature drying of the slides to be detected without lifting the lid.

Table 1 Procedure for immunoperoxidase staining.

STEP	1	2	3	4	5
REAGENT	H_2O_2	WASH	WASH	NSS	Specific antibody
DILUTION	3%			1:5	Variable
DILUANT	Methanol	H_2O	2% NSS TBS	TBS	2% NSS TBS
TIME	30 min	5 min	5 min	10 min	30 min

STEP	6	7	8	9	10
REAGENT	WASH	SAR	WASH	PAP	WASH
DILUTION		1:30		1:100	
DILUANT	2% NSS TBS				TBS
TIME	15 min	30 min	15 min	30 min	15 min

STEP	11	12	13	14	15
REAGENT	AEC	WASH	Haema-toxylin	WASH	Mount in glycerine jelly
DILUTION	1:10				
DILUANT	Acetate buffer + 1 drop 30 vols H_2O_2	H_2O		H_2O	
TIME	5 min	5 min	5 min	5 min	

Notes for Table 1

NSS - Normal swine serum[i]

TBS - Tris buffered saline pH 7.6[ii]
(diluted 1:10 with 0.85% saline before use)

SAR - Swine anti-rabbit immunoglobulin[iii]

PAP - Rabbit anti-peroxidase peroxidase conjugate[iv]

AEC - Aminoethyl carbazole[v]
(0.4%) in dimethyl formamide

NB When formalin-fixed tissue sections were being examined rather than cytocentrifuge preparations, they were placed in 0.1% trypsin in 0.1% $CaCl_2$ in distilled water (pH 7.6 - 7.8) for 15 minutes at 37°C. This was done between steps 2 and 3, and was followed by a 10 minute wash in water.

i. NSS - courtesy of FMC abattoir, Newmart Road, Edinburgh

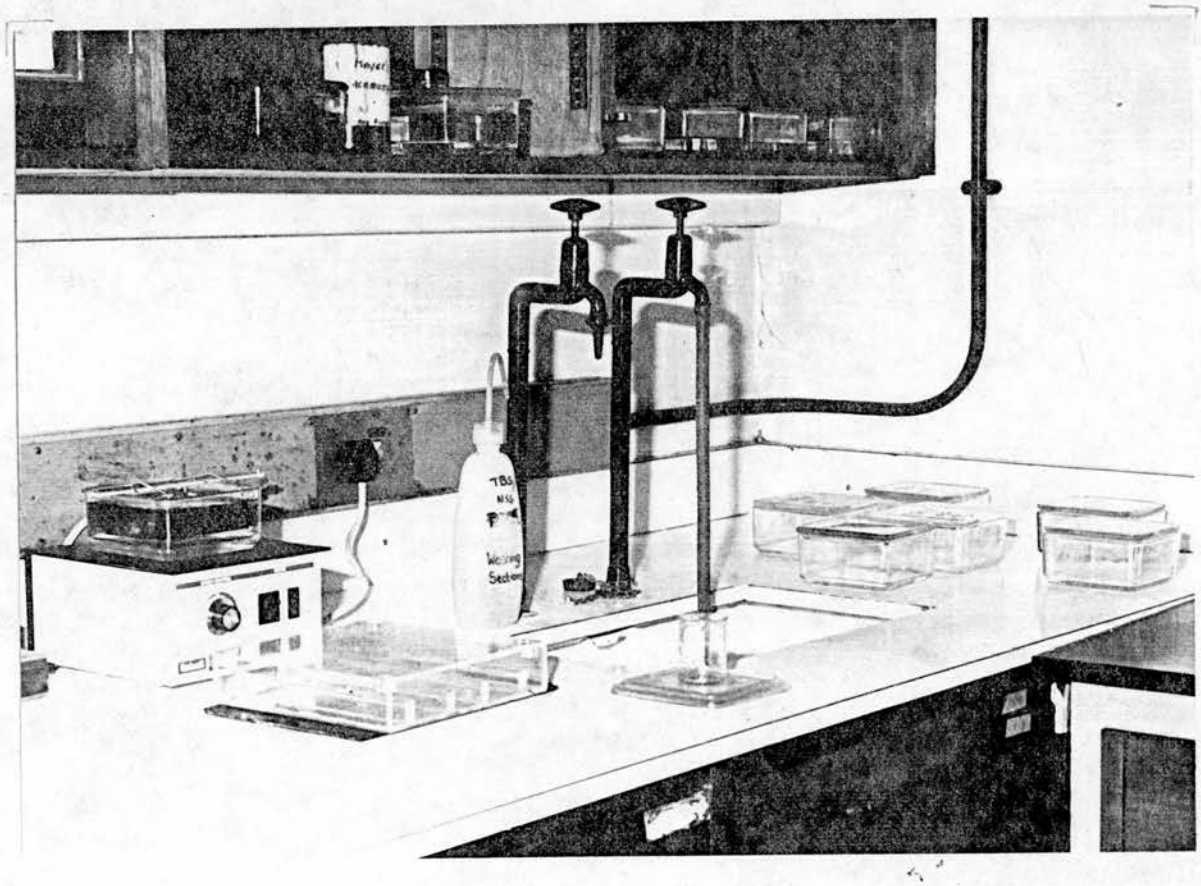
ii. TBS - Tris 6.05 g
1/N HCl 40 mls
A.D. 960 mls pH 7.4
NaCl 8.5 g
Tris (hydroxymethyl) methylamine. FISONS

iii. SAR - DAKO Z196

iv. PAP - DAKO Z113

v. AEC - Aminoethyl carbazole (0.4%) in dimethyl formamide was made up 1:10 in acetate buffer with one drop of H_2O_2 (30 vols) just before use.
Acetate buffer: M/1 sodium acetate 50 mls
N/1 HCl 10 mls
A.D. 200 mls

Figure 9 Equipment used for immunoperoxidase assay.



1 x. Statistical methods

In most instances, non-parametric methods were used to analyse the data, and the test used in each individual case is described in the text, along with the probability value obtained.

The tests used were:

a) Wilcoxon's rank sum test

b) Wilcoxon's signed rank test for paired data

c) Kendall's rank correlation test

d) Student's t test

e) Cox's procedure for combining several regressions which have a binary response

The first four of these tests are described in Part 1 of this thesis (Volume 1, pages 126 - 127), and Cox's procedure is referenced in the text.

2. Collagenase control experiments

Introduction

As described in the general methods section, both tumours and lymph nodes had to be incubated in collagenase in order to release sufficient numbers of macrophages for study (q.v. pages 63 and 65). It was therefore necessary to establish whether the collagenase preparation used had any effect on the macrophage parameters which were being measured in the experiments; namely, lysozyme and alpha-1-antitrypsin content, Fc and C3 receptor expression, the ability to phagocytose IgG-coated erythrocytes, and the presence of surface antigens detected by the anti-macrophage (My6C3) and the anti-Ia (BT2/9) monoclonal antibodies.

Method

Mononuclear cells were isolated from the blood of 10 healthy volunteers using a Ficoll-Hypaque gradient (q.v. pages 60 - 62). 10^7 cells were then incubated with 15 mls. of collagenase for 12 hours, washed six times in medium with 2% FCS and resuspended in 10% FCS. This treatment resulted in a monocyte loss of 10-20%.

Firstly, lysozyme and alpha-1-antitrypsin immunoperoxidase assays (q.v. pages 78 - 83) were carried out on cytocentrifuge preparations of treated and untreated cells. Secondly, the following monocyte surface markers were determined by rosetting reactions (q.v. pages 66 - 74) on treated and untreated cells:

1) Fc receptors 2) C3 receptors

3) My6C3 antigen 4) Ia antigen

Cytocentrifuge preparations of these rosetted cells were then made and stained with haematoxylin so that the percentage of monocytes forming rosettes could be ascertained. Thirdly, the effect of collagenase on the ability of monocytes to phagocytose IgG-coated ox erythrocytes during a period of 2 hours at 37°C was examined.

Results

Twelve hours of collagenase treatment at 37°C did not alter the percentage of monocytes containing either lysozyme or alpha-1-antitrypsin (Figures 10 and 11). However, collagenase did have a slight enhancing effect on Fc receptor expression (Figure 12) and a very marked depressing effect on C3 receptor expression (Figure 13). In addition, there was a marginal decrease in the percentage of monocytes reacting with the My6C3 monoclonal antibody (Figure 14), but no change in the frequency of Ia antigen expression (Figure 15). Finally, no significant effect on the percentage of Fc receptor bearing monocytes capable of phagocytosing the IgG-coated erythrocytes after incubation at 37°C for 2 hours was observed (Figure 16).

Discussion

Fortunately, collagenase does not appear to alter the content of lysozyme or alpha-1-antitrypsin in monocytes, and it is therefore reasonable to assume that the enzymatic method used to obtain macrophages from tumours and lymph nodes

Figure 10 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes containing lysozyme ($P > 0.1$ by Wilcoxon's signed rank test for paired data).

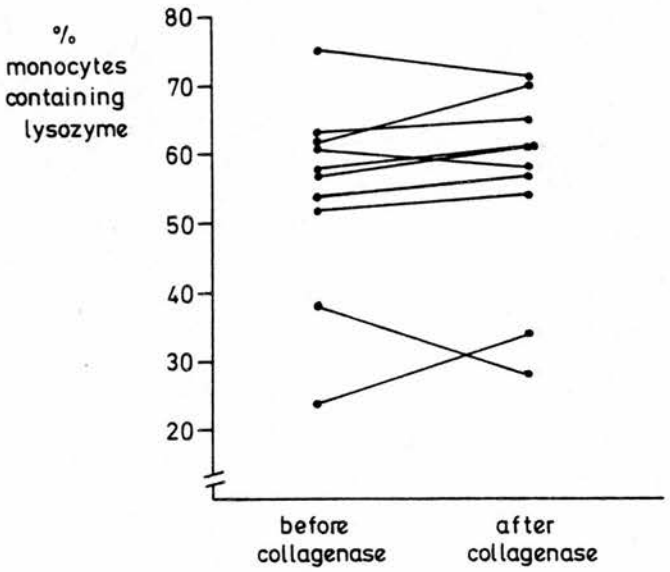


Figure 11 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes containing alpha-1-antitrypsin ($P > 0.1$ by Wilcoxon's signed rank test for paired data).

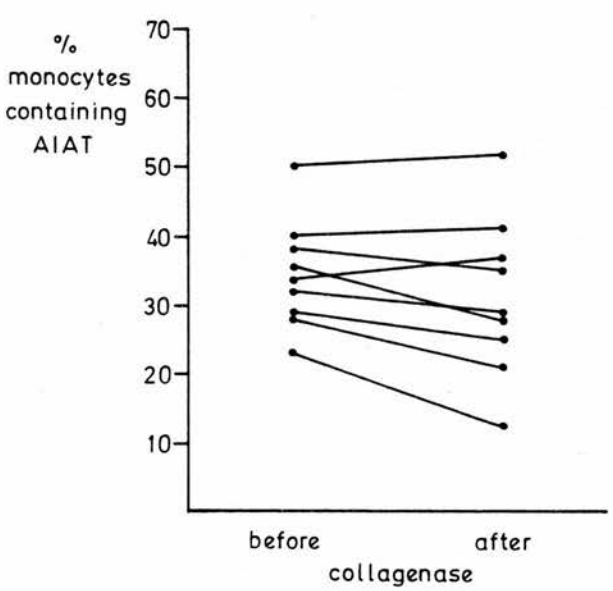


Figure 12 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes bearing Fc receptors ($P < 0.01$ by Wilcoxon's signed rank test for paired data).

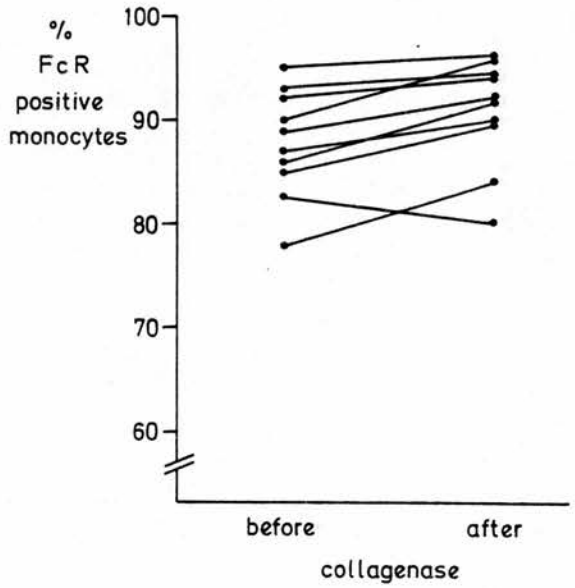


Figure 13 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes bearing C3 receptors ($P < 0.01$ by Wilcoxon's signed rank test for paired data).

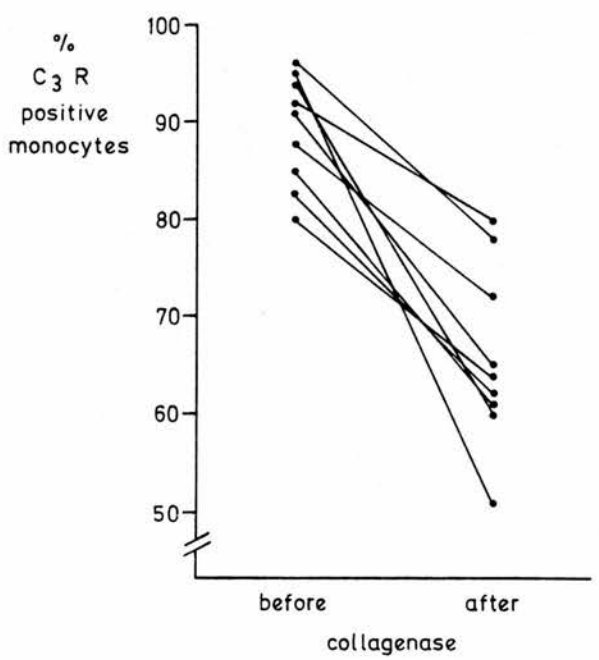


Figure 14 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes reacting with My6C3 ($P < 0.01$ by Wilcoxon's signed rank test for paired data).

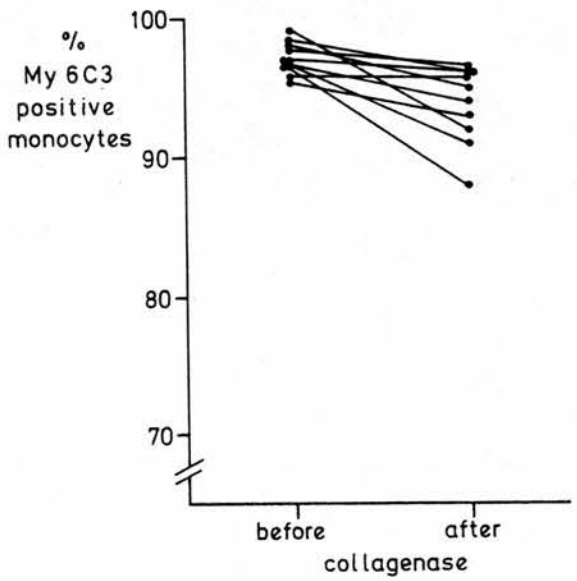


Figure 15 The effect of 12 hours incubation in collagenase at 37°C on the percentage of monocytes expressing Ia surface antigen ($P > 0.1$ by Wilcoxon's signed rank test for paired data).

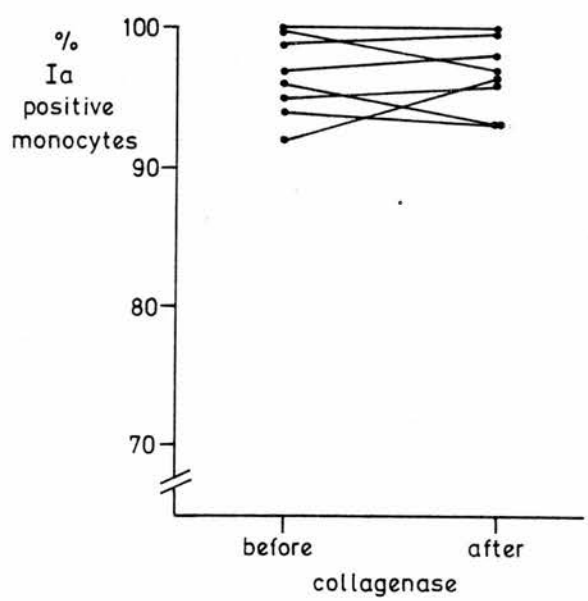
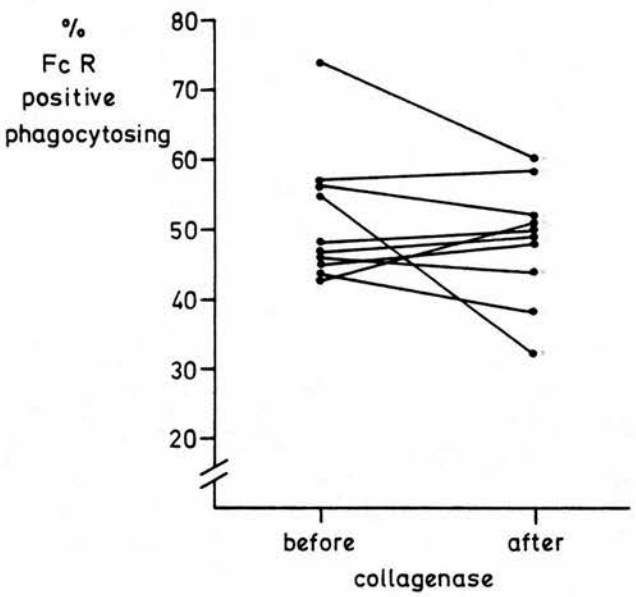


Figure 16 The effect of 12 hours incubation in collagenase at 37°C on the percentage of Fc receptor-positive monocytes capable of phagocytosing the IgG-coated red cells.



(q.v. pages 63 and 65) will not affect these parameters. On the other hand, some of the surface characteristics of monocytes are susceptible to collagenase treatment. Fc receptor activity seems to be slightly increased, and the most likely explanation for this phenomenon is increased receptor availability due to removal of surface immunoglobulin or immune complexes. Trypsin has been shown to have a similar action[170], and the present effect may be due to small amounts of this enzyme in the collagenase preparation (q.v. page 63). In contrast to the Fc receptor, the C3 receptor fares rather badly in the face of collagenase, and appears to be damaged or stripped off to a marked extent. Again, this may be due to trypsin contamination, as trypsin is known to decrease C3 receptor activity[180,187]. The monoclonal antibody My6C3 probably identifies a differentiation antigen on the surface of monocytes, and this antigen seems to be slightly less prominent after collagenase treatment. The Ia antigen, however, does not appear to be affected.

As a result of these experiments, it is clear that C3 receptor expression cannot be accurately measured after collagenase treatment. The alterations in Fc receptor expression and reaction with the My6C3 antibody, however, were slight, and after enzyme treatment both remain useful monocyte markers.

3. Evidence that tumour cells do not bear Fc receptors, My6C3 antigen or Ia antigen

Introduction

In cell suspensions from tumours, it is virtually impossible to distinguish macrophages from tumour cells on morphological grounds alone, as both may have abundant cytoplasm and irregular nuclei. For this reason, it is necessary to use some form of marker to identify macrophages in tumour cell suspensions, and, in the studies described in this thesis, three separate markers have been employed. Firstly, Fc receptors were detected by a rosetting reaction using IgG-coated ox erythrocytes (EA). Secondly, a mouse anti-human macrophage monoclonal antibody (My6C3) coupled to sheep erythrocytes was used, and thirdly, another mouse monoclonal antibody, again coupled to red cells, was employed to detect immune response associated (Ia) antigen on the cell surface.

However, despite the careful use of controls, it is not possible to guarantee that tumour cells cannot display these markers. Accordingly, experiments were carried out using rabbit anti-human epithelial membrane antigen (EMA) as a marker for breast cancer cells in order to determine whether tumour cells could exhibit Fc receptors, My6C3 antigen or Ia antigen.

Method

Cell suspensions from 13 human breast cancers were prepared by mechanical disaggregation and collagenase digestion (q.v. pages 63 - 64). Rosettes were then formed (q.v. pages

74 - 75) using the following individual indicator erythrocytes: 1. Ox erythrocytes coated with rabbit IgG against ox erythrocytes to detect Fc receptors (q.v. pages 66 - 67).

2. Sheep erythrocytes coupled to My6C3 monoclonal antibody to detect the differentiation antigen on macrophage cell surfaces (q.v. pages 69 - 72).

3. Sheep erythrocytes coupled to BT2/9 monoclonal antibody to detect the Ia antigen on macrophage cell surfaces (q.v. pages 69 - 72).

Cytocentrifuge preparations of these rosetted cells were then made, and the immunoperoxidase assay (q.v. pages 78 - 83) was carried out. The specific antiserum used at step 5 of the assay (q.v. page 81) was rabbit anti-human EMA[i], so that positive staining indicated the presence of EMA in an individual cell.

In each case, approximately 100 non-lymphocytic cells were counted; a note was taken of how many had formed rosettes, and the numbers of EMA-positive cells in the rosetted and non-rosetted populations were also recorded.

i. The rabbit anti-human EMA was kindly donated by Dr. M.G. Ormerod, Institute of Cancer Research.

Results

Examples of rosetted preparations are shown in Figures 17 - 20, and the overall results are displayed in Figures 21 - 23. Essentially, they demonstrate that rosette forming cells were very rarely positive for EMA, the percentages ranging from 0 - 5% of rosetted cells. As the rate of rosette formation in control preparations was 1 - 2% anyway, this figure is probably within the limits of experimental error. On the other hand, non-rosetting cells were positive for EMA in a variable but usually fairly high percentage of cases (8 - 68%). With all three markers, this association between EMA and the absence of rosette-forming ability was highly significant by Cox's procedure for combining several regressions which have a binary response[294].

Discussion

As previously mentioned, macrophages require to be distinguished from tumour cells by means other than morphological identification, and in the present studies, Fc receptor expression, an anti-macrophage monoclonal antibody (My6C3), and an anti-Ia monoclonal antibody have been used. Theoretically, these should be suitable macrophage markers, because, although some lymphocytes have Fc receptors (q.v. page 26), B-lymphocytes express Ia antigens (q.v. page 33), and the present studies indicate that about 5% of lymphocytes share the My6C3 antigen, lymphocytes can be distinguished from macrophages morphologically.

Figure 17 A rosetted preparation of a breast tumour cell suspension using the My6C3 antibody. Non-rosetting EMA-positive (red) cells are clearly seen in the lower right hand corner. (x 50)

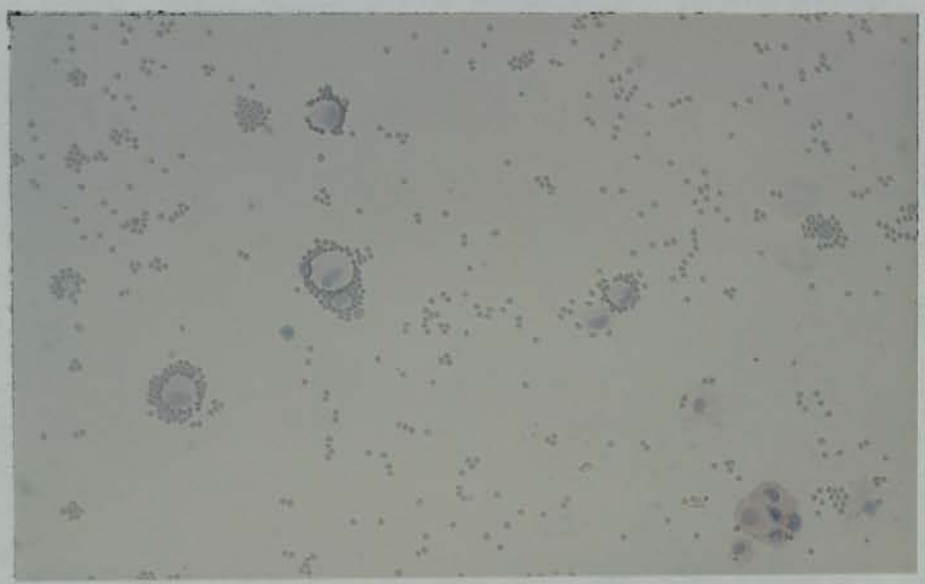


Figure 18 A preparation of a breast tumour cell suspension using EA rosettes to detect Fc receptors. The EMA-positive (red) cells are not rosetting. (x 50)

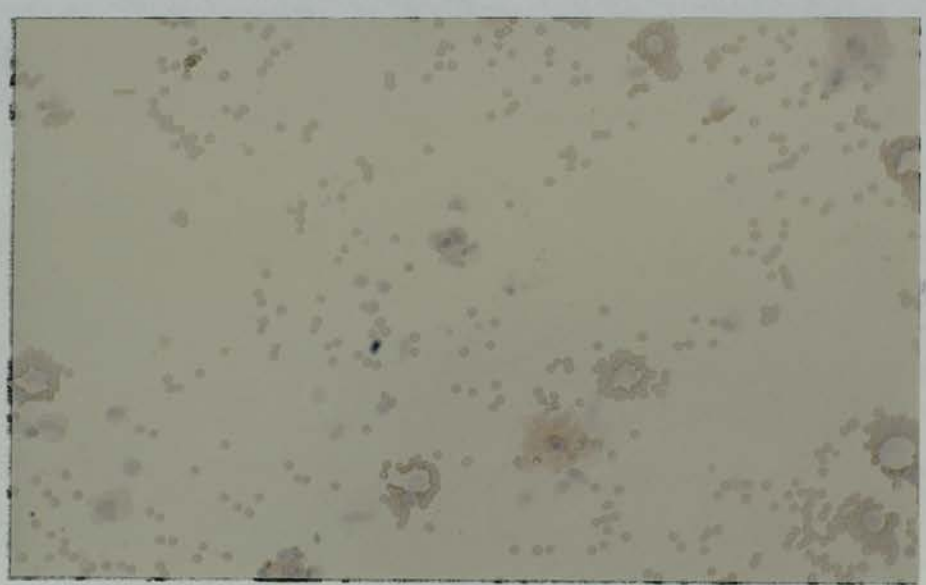


Figure 19 A preparation of a breast tumour cell suspension using EA rosettes to detect Fc receptors. The EMA-positive (red) cells are not rosetting. Note that the erythrocytes stain red because they are coated with rabbit immunoglobulin. (x 160)

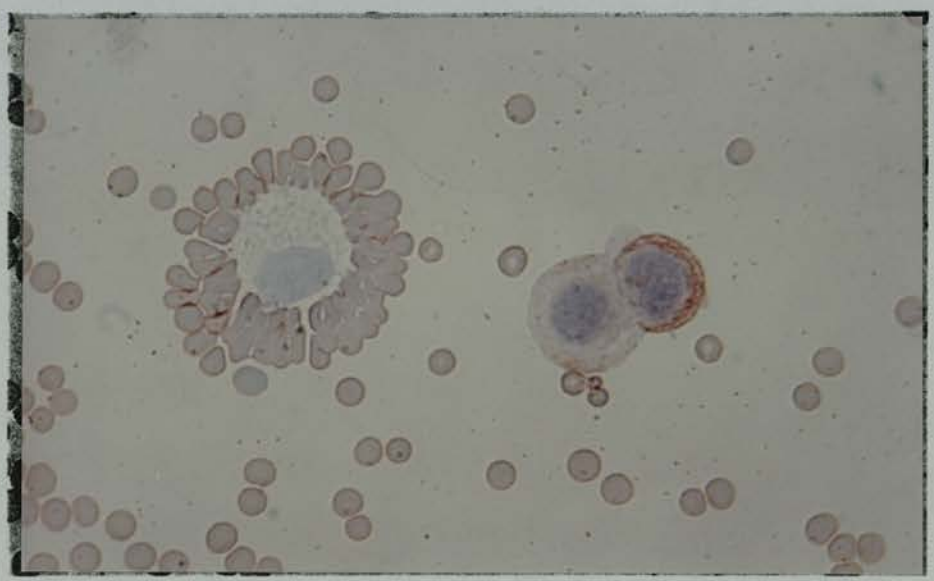


Figure 20 A rosette preparation of a breast tumour cell suspension using anti-Ia (BT2/9) antibody. The EMA-positive (red) cell is not rosetting. (x 160)

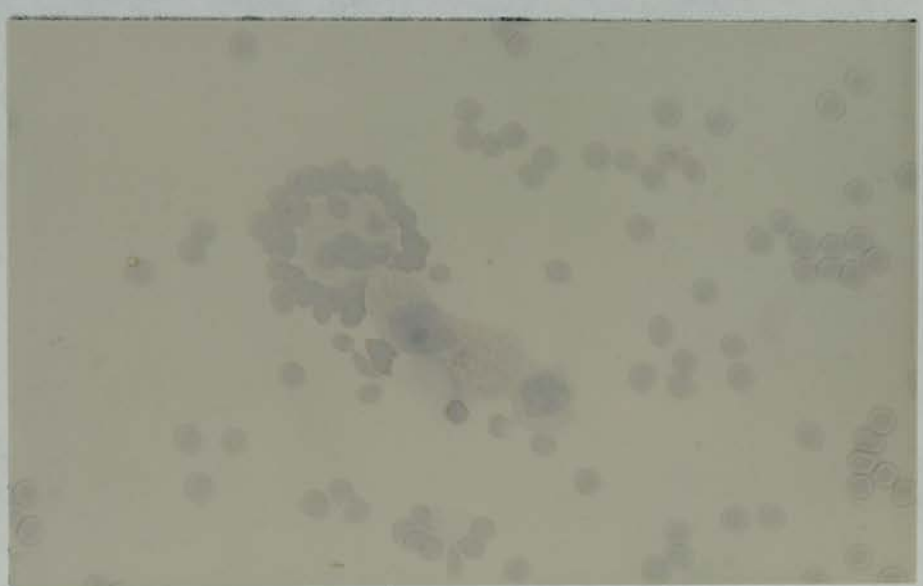


Figure 21 The percentage of Fc receptor-positive and Fc receptor-negative cells displaying EMA in 13 individual breast cancer cell suspensions. The figures above each column indicate the actual number of cells counted (denominator) and the number positive for EMA (numerator). ($P < 0.001$ by Cox's procedure.)

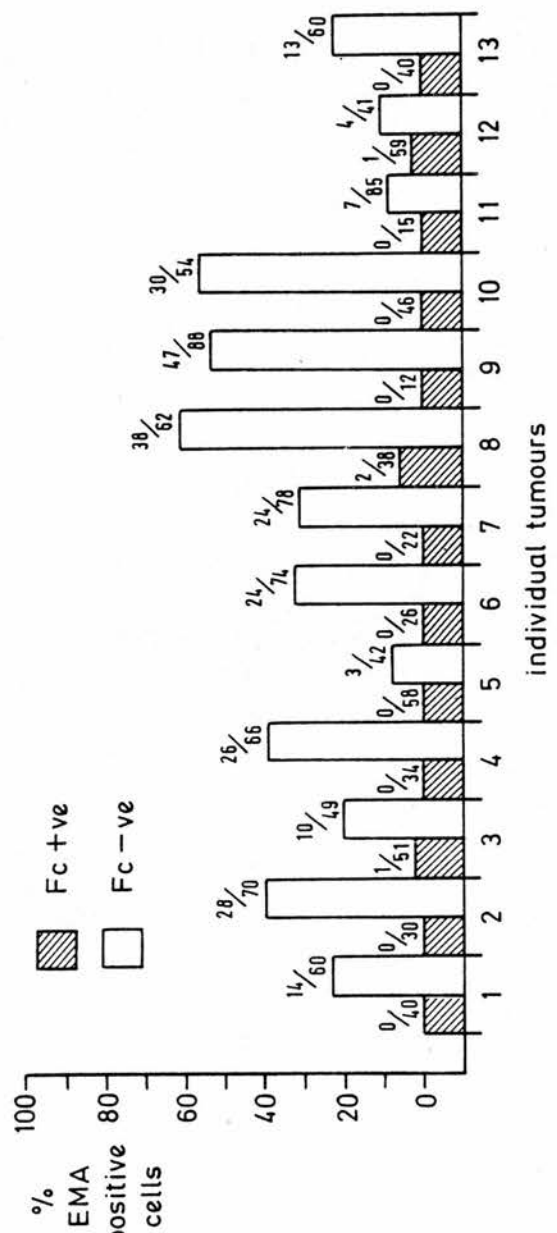


Figure 22 The percentage of My6C3-positive and My6C3-negative cells displaying EMA in 13 individual breast cancer cell suspensions. The figures above each column indicate the actual number of cells counted (denominator) and the number positive for EMA (numerator).
($P < 0.001$ by Cox's procedure.)

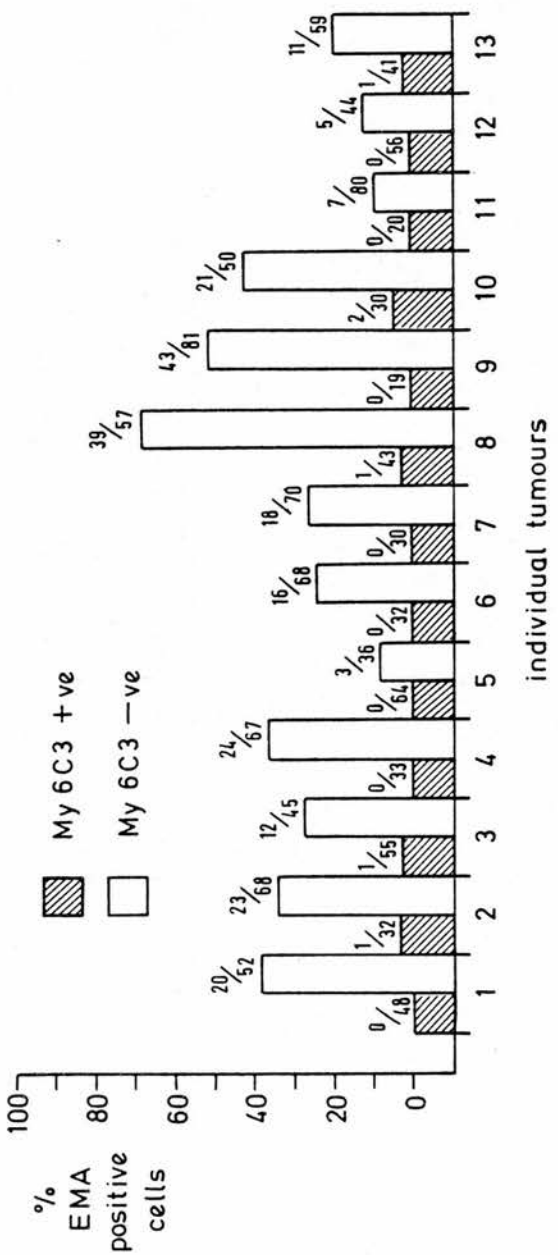
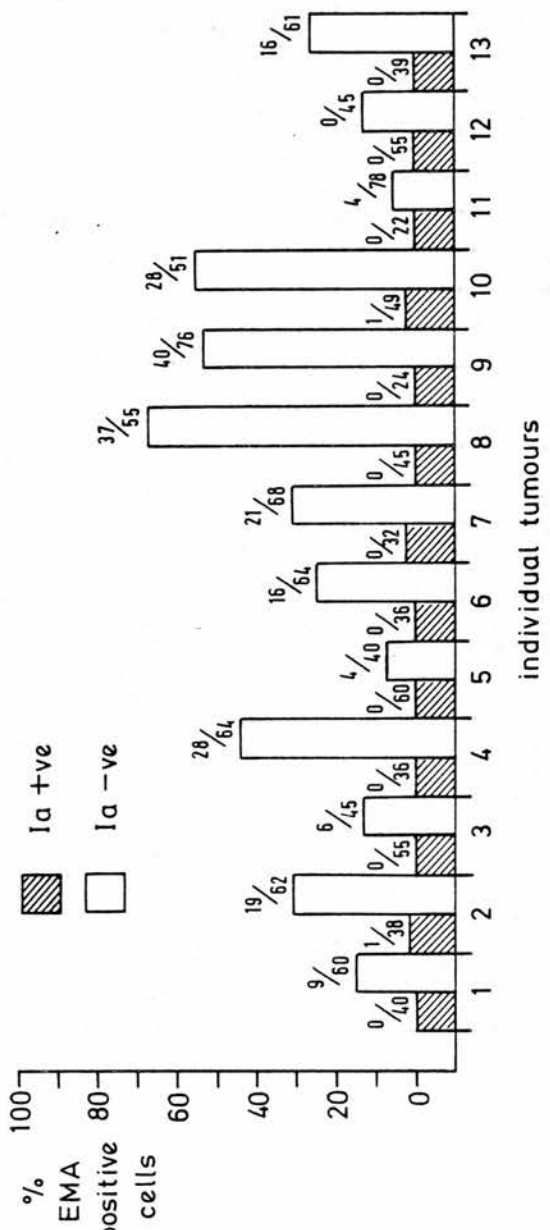


Figure 23 The percentage of Ia-positive and Ia-negative cells displaying EMA in 13 individual breast cancer cell suspensions. The figures above each column indicate the actual number of cells counted (denominator) and the number positive for EMA (numerator).
($P < 0.001$ by Cox's procedure.)



However, for these markers to be useful, it is necessary to establish that they do not appear on tumour cells. There is fairly good evidence that tumour cells do not have Fc receptors (q.v. pages 6 - 7), although it is better to have extra supporting evidence for any individual system. However, the ability of My6C3 to react with tumour cells has not been investigated, and it has not been firmly established whether Ia antigens can appear on the surface of neoplastic cells.

In this present study, localisation of cell-surface components (EMA) using rabbit antiserum raised against human milk-fat-globule membranes[295] has been employed to identify breast tumour cells in cytocentrifuge preparations of cell suspensions in which macrophages are putatively marked by rosette formation. EMA was chosen because it is expressed by nearly all breast cancers[296]. However, it cannot be demonstrated in all the neoplastic cells within a single tumour[295], it is not exclusive to neoplastic cells, and it is not even exclusive to breast-derived cells[297]. On the other hand, EMA only appears to be present in cells of epithelial origin[297], so that in a cell suspension from a breast tumour, host macrophages should not display the antigen, and EMA positive cells can be assumed to be tumour cells with a fair degree of certainty.

The results of this study demonstrate that EMA positive cells do not carry Fc receptors, My6C3 antigen or Ia antigen, strongly suggesting that tumour cells do not bear these markers.

4. Lysozyme content of breast cancer-associated macrophages

Introduction

Lysozyme is a major proteolytic enzyme which is found in macrophages, and which is in fact synthesised by them (q.v. pages 38 - 39). It was decided to study the lysozyme content in breast cancer-associated macrophages for two reasons; firstly, it provides a measure of macrophage activation (q.v. pages 39 - 40), and secondly, there is evidence that lysozyme can damage tumour cells[246]. To carry out these investigations, it was elected to obtain blood monocytes, macrophages from tumour-draining axillary lymph nodes and tumour-infiltrating macrophages from breast cancer patients, and to compare these with monocytes and nodal macrophages from comparable controls.

Method

a) Patients

16 patients with breast cancer clinically confined to the breast and axilla were studied, and venous blood and primary tumours were obtained. From 12 of these patients, axillary lymph nodes, which were subsequently confirmed to be free of tumour by histological examination, were also available. Venous blood was also obtained from 14 female control patients who were about to undergo surgery for non-neoplastic, non-inflammatory conditions, and lymph nodes were obtained from various sites in 10 similar patients during surgery. Details of all these patients are given in the appendix.

b) Preparation of cells

Blood mononuclear cells were isolated using a Ficoll-Hypaque gradient as previously described (q.v. pages 60 - 62), and cell suspensions from lymph nodes and tumours were prepared by collagenase digestion (q.v. pages 63 - 65).

c) Macrophage surface markers

Macrophage surface markers were determined in the various cell suspensions by 3 separate rosetting assays. Fc receptors were detected by EA rosettes (q.v. pages 66 and 74). Macrophage-associated antigen was detected by the My6C3 mouse monoclonal antibody coupled to sheep erythrocytes (q.v. pages 69 - 74), and Ia surface antigen was displayed by the BT2/9 (anti-Ia) mouse monoclonal antibody, also coupled to sheep erythrocytes (q.v. pages 69 - 74). Controls for the My6C3 and Ia indicators, comprising sheep erythrocytes coupled to normal mouse immunoglobulin, were used to establish that non-specific Fc binding was not occurring.

d) Lysozyme assay

Lysozyme was detected on cytocentrifuge preparations (q.v. pages 75 - 76) of these rosetted cell suspensions by means of the PAP immunoperoxidase method (q.v. pages 78 - 83), and the percentage of rosetting cells containing lysozyme was estimated by counting 200 cells. The antiserum used was rabbit anti-human lysozyme immunoglobulin fraction (DAKO A099) diluted 1 in 400 with 5 mls. of Tris-buffered saline containing 2% normal swine serum.

As a control, a similar amount of test antiserum was incubated with 100 µg. of human placental lysozyme (Alpha Therapeutic Corporation) for 1 hour at room temperature before use. This procedure consistently abolished the immunoperoxidase staining. Positive controls consisting of a series of cytocentrifuge preparations of blood monocytes from one donation were used, and similar percentages of lysozyme-positive cells were observed in each individual immunoperoxidase run.

e) Tissue sections

In order to determine whether the percentages of lysozyme-containing cells from the cell suspensions produced by collagenase digestion were comparable to the actual percentages of lysozyme-containing cells in the tumours or lymph nodes, formalin-fixed sections of these tissues were also subjected to the immunoperoxidase assay. The procedure differed from that employed for the cytocentrifuge preparations only in that the sections were trypsin-treated before the assay was performed (q.v. page 82).

f) Prognostic factors

The percentages of monocytes, nodal macrophages and tumour-infiltrating macrophages containing lysozyme were compared to the four prognostic factors of lymph node status, oestrogen receptor status, tumour size and tumour grade (q.v. pages 58 - 59). This was done to ascertain whether any relationship existed between macrophage lysozyme content and prognosis in cancer patients.

Results

Examples of cytocentrifuge preparations of the rosetted cells stained for lysozyme by the PAP method are shown in Figures 24 - 30, and the overall results are displayed in Figures 31 - 33. All three macrophage markers gave very similar results, which can be summarised as follows.

a) Blood monocytes

Monocytes from the cancer patients contained lysozyme in significantly greater numbers than did monocytes from the control patients ($P < 0.001$ by Wilcoxon's rank sum test for the Fc receptor and My6C3 markers, and $P < 0.01 > 0.001$ for the Ia marker).

b) Nodal macrophages

Macrophages from nodes draining breast tumours did not differ significantly from macrophages from control nodes in terms of lysozyme content ($P > 0.1$ by Wilcoxon's rank sum test for each marker). However, both control nodal macrophages and tumour nodal macrophages contained lysozyme significantly less frequently than control or tumour blood monocytes respectively ($P < 0.001$ by Wilcoxon's rank sum test for each marker).

c) Tumour infiltrating macrophages

The tumour infiltrating macrophages contained lysozyme in low numbers, the enzyme appearing significantly less frequently than in the blood monocytes from the cancer patients ($P < 0.001$ by Wilcoxon's rank sum test for each

Figure 24 Blood monocyte forming a rosette with My6C3-coated erythrocytes and staining positively for lysozyme. (x 160)

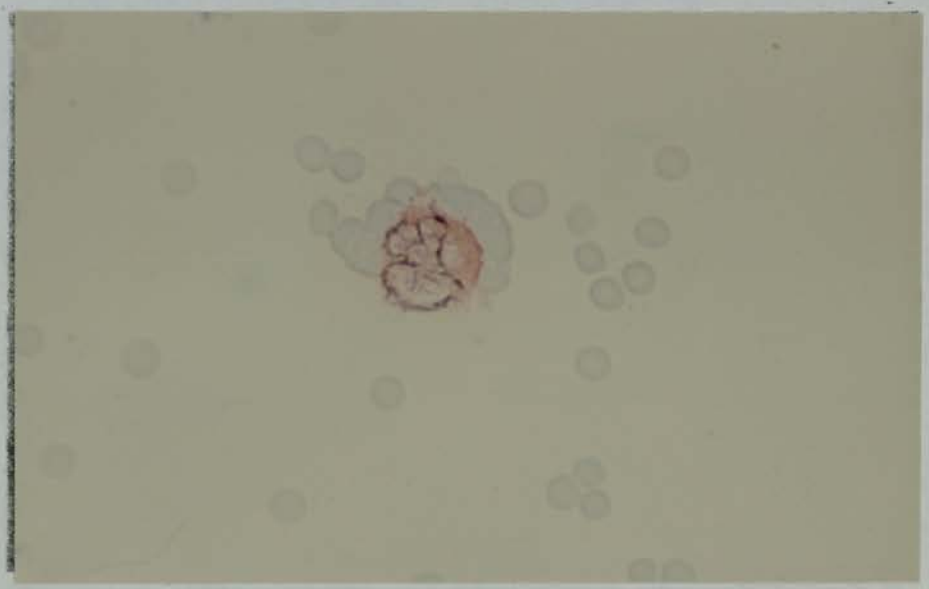


Figure 25 Blood monocyte forming an Fc-receptor detecting (EA) rosette, and staining positively for lysozyme. Lymphocyte in lower part of field is not stained. (x 160)

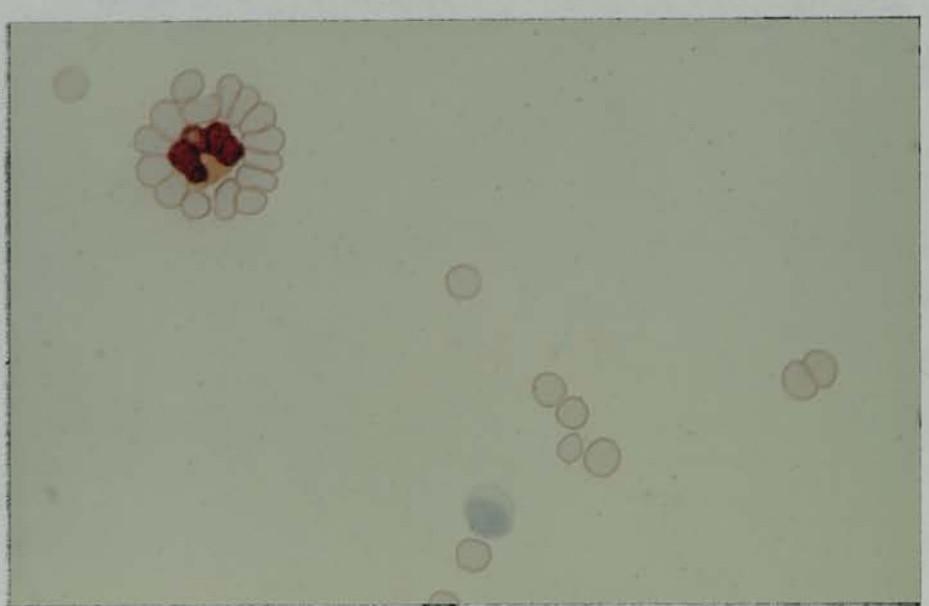


Figure 26 Nodal macrophage forming an Fc-receptor detecting (EA) rosette, but not staining for lysozyme. (x 160)

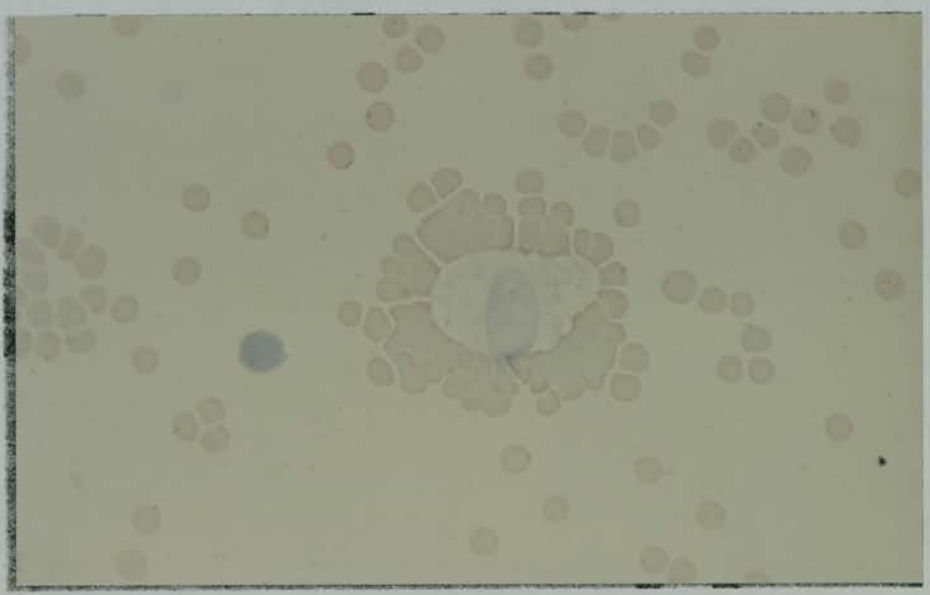


Figure 27 Nodal macrophage forming an Ia-detecting rosette, and staining positively for lysozyme. (x 160)

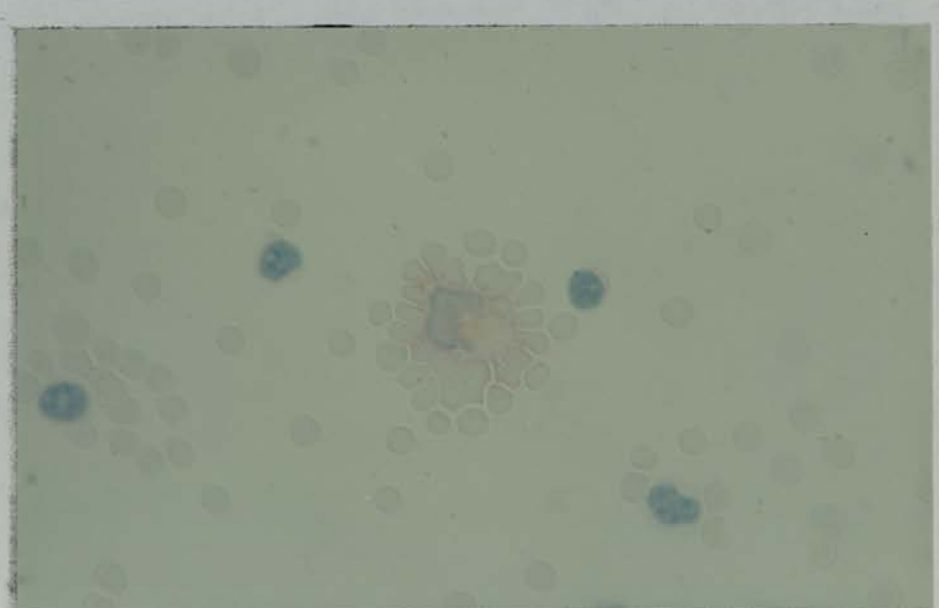


Figure 28 Two tumour-infiltrating macrophages forming Fc-receptor detecting (EA) rosettes, but both negative for lysozyme. (x 160)

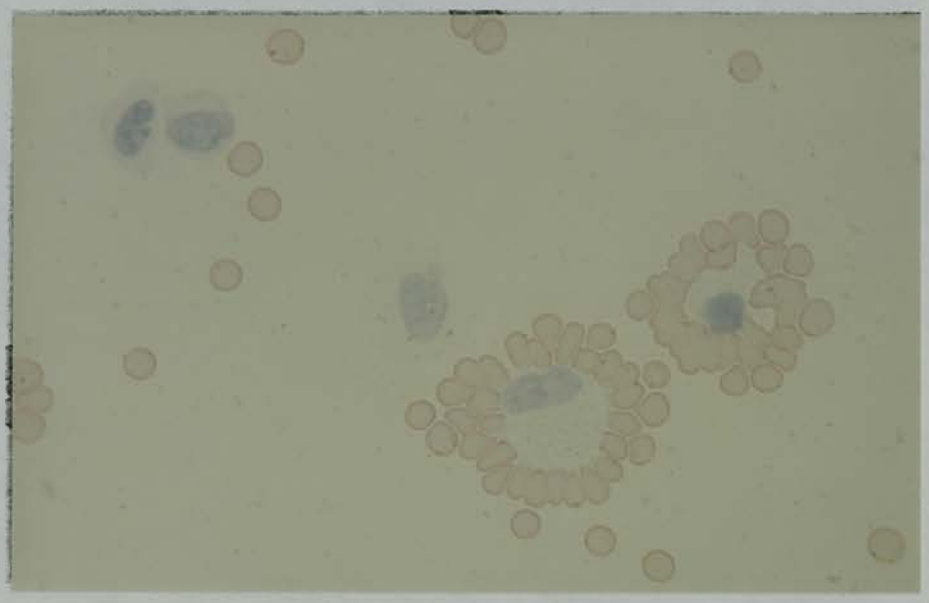


Figure 29 Three tumour-infiltrating macrophages forming rosettes with My6C3-coated erythrocytes. One staining weakly for lysozyme. (x 160)

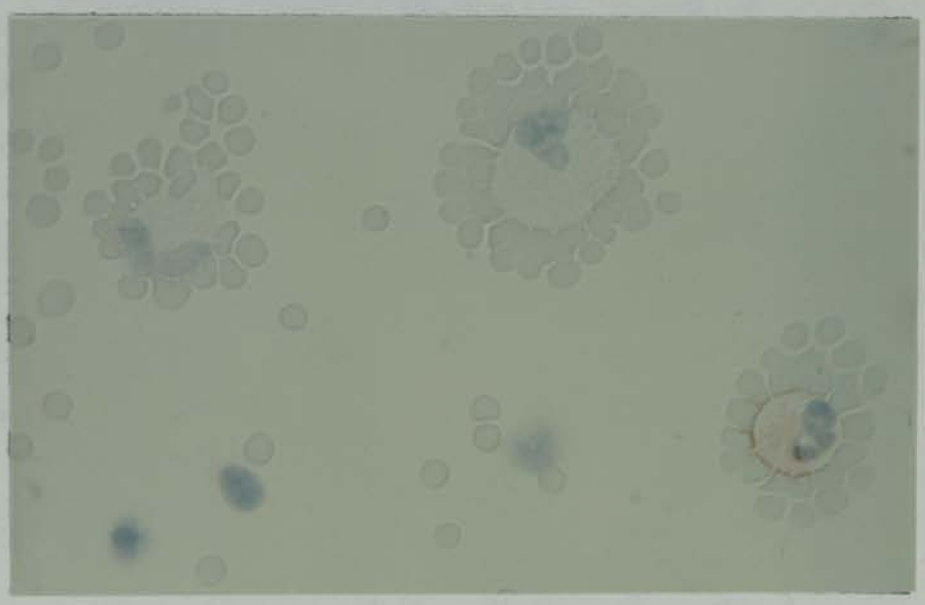


Figure 30 Tumour-infiltrating macrophage forming an Ia detecting rosette, but not staining positively for lysozyme. (x 160)

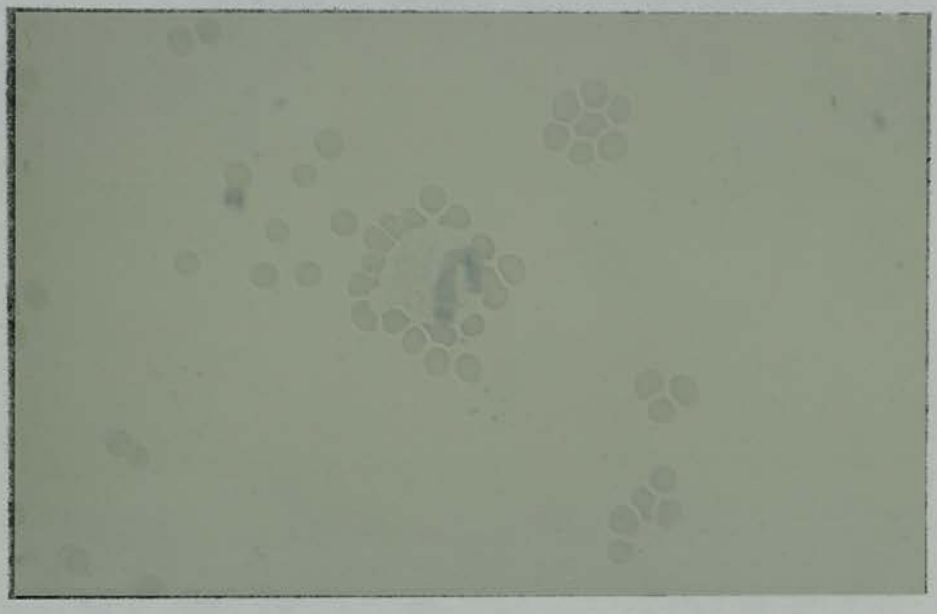


Figure 31 Percentages of Fc receptor positive cells of the monocyte/macrophage series containing lysozyme.

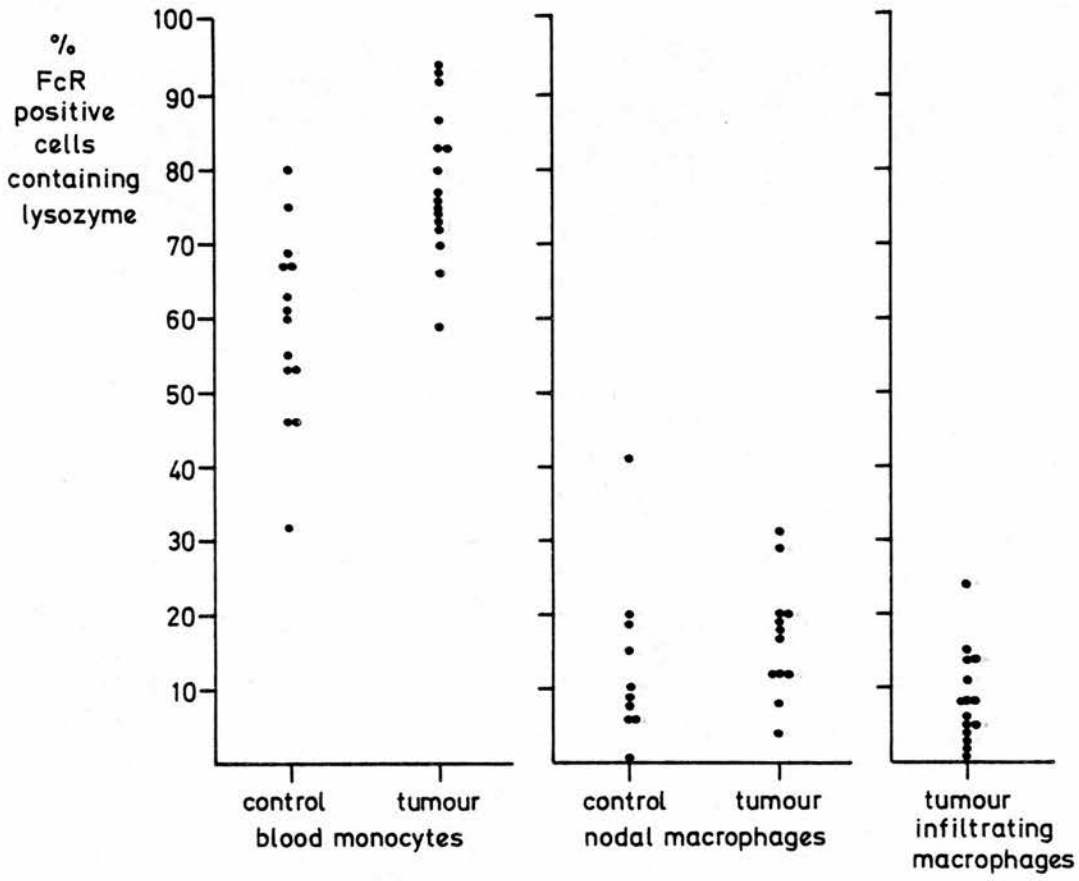


Figure 32 Percentages of My6C3 positive cells of the monocyte/macrophage series containing lysozyme.

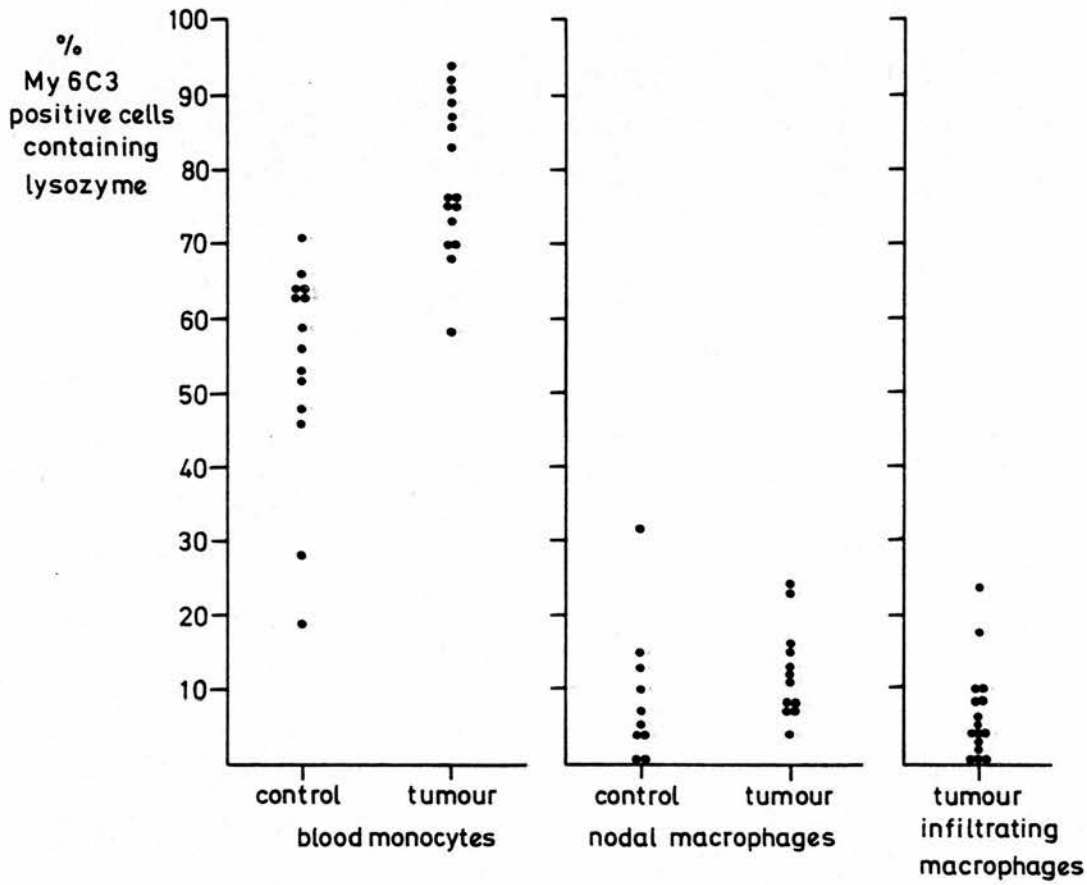
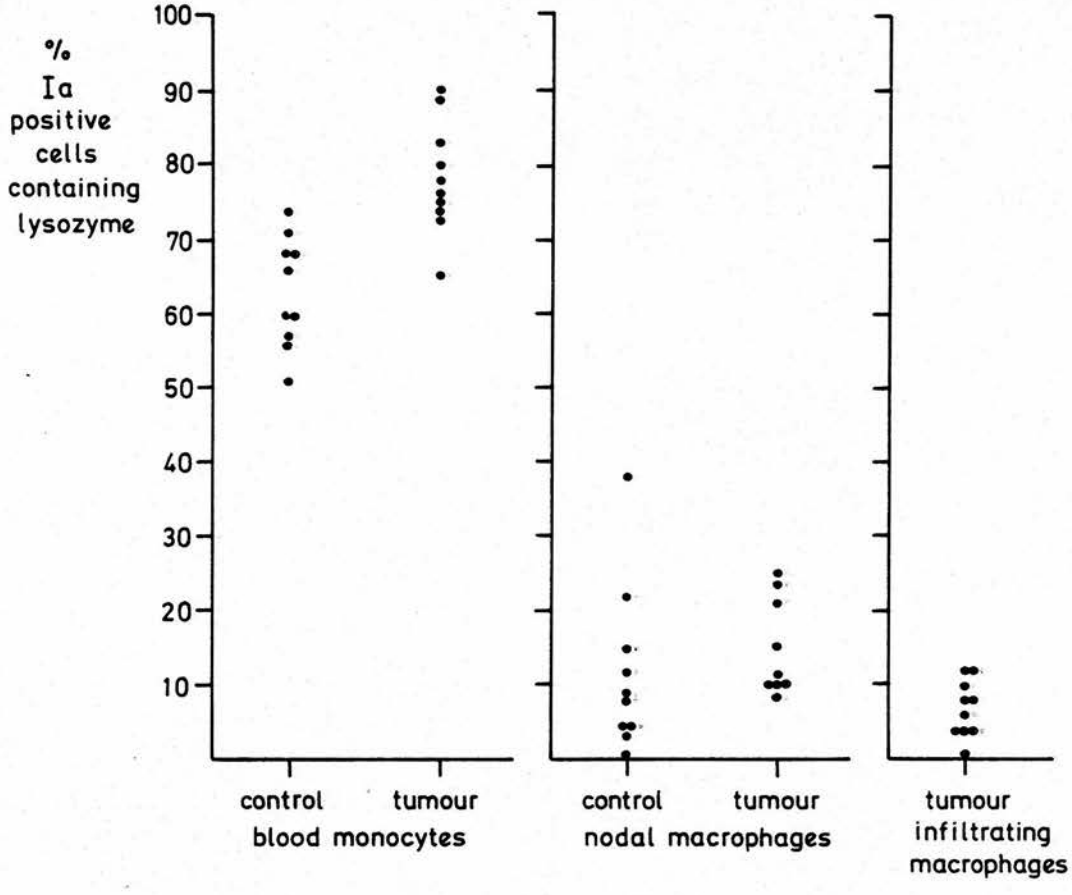


Figure 33 Percentages of Ia positive cells of the monocyte/macrophage series containing lysozyme.



marker). In addition, when the data was paired, the tumour-infiltrating macrophages were positive for lysozyme significantly less often than tumour nodal macrophages ($P < 0.05$ by Wilcoxon's signed rank test for each marker).

d) Tissue sections

Immunoperoxidase staining for lysozyme on tissue sections of tumour and lymph node is illustrated in Figures 34 and 35. A comparison between the percentages of lysozyme-positive cells in non-rosetted cytocentrifuge preparations of cell suspensions from tumours and nodes and the percentages of lysozyme positive cells on sections of these tissues is shown in tables 2 and 3. As can be seen, the figures are reasonably comparable, suggesting that collagenase disaggregation of tumours and nodes does not substantially alter the percentage of cells containing lysozyme, although it is known that some cell loss does occur (q.v. page 85).

e) Prognostic factors

No relationship between macrophage lysozyme content and any of the prognostic factors examined was found to exist.

Discussion

In this study, tumour infiltrating macrophages have been identified using three separate rosetting reactions, and lysozyme content of these cells has been assessed by an immunoperoxidase assay. The assay has also been used to

Figure 34 Tissue section of invasive breast cancer immunostained for lysozyme (red) and counter stained with haematoxylin (blue).

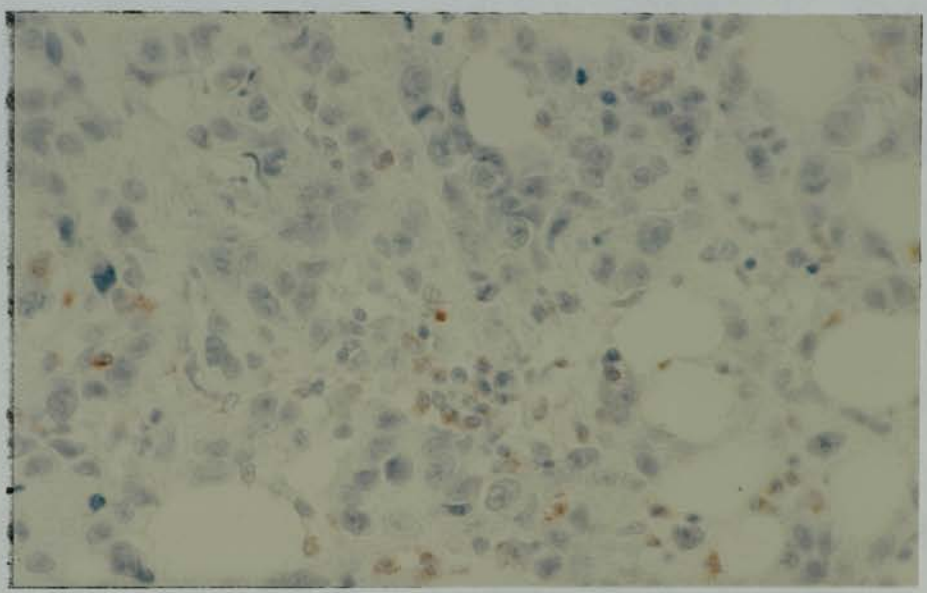


Figure 35 Tissue section of breast tumour-draining lymph node immunostained for lysozyme (red).

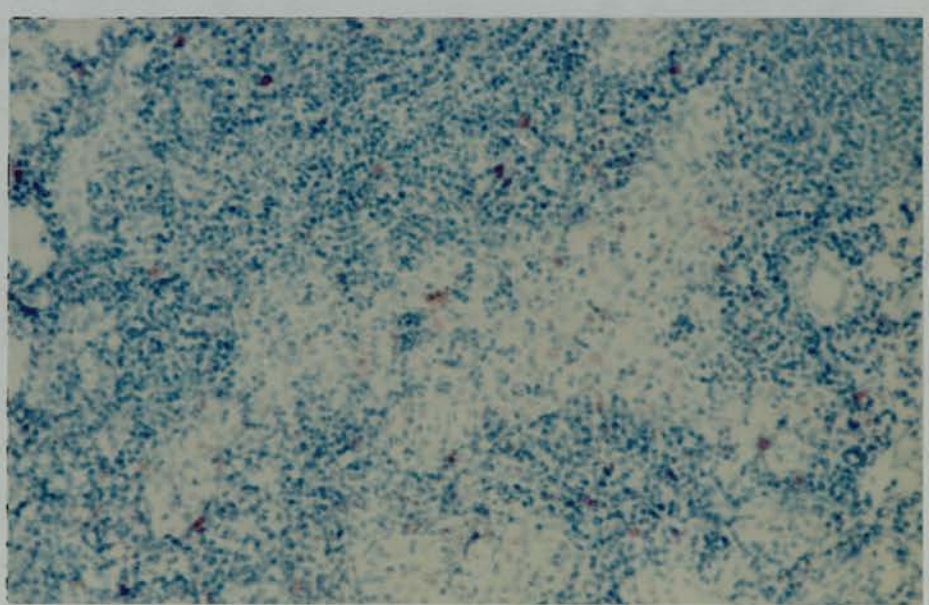


Table 2 Comparison of the percentages of the TOTAL CELL POPULATION containing lysozyme in cytocentrifuge preparations from tumour cell suspensions, and in tissue sections.

% cells positive for lysozyme	
Cell suspension	Tissue section
6	8
<1	<1
2	<1
1	<1
2	1
3	<1
2	3
4	6
<1	<1
10	27
<1	2
<1	<1
5	3
4	<1

Table 3 Comparison of the percentages of the TOTAL NON-LYMPHOCYTIC CELL POPULATION containing lysozyme in cytocentrifuge preparations from lymph node cell suspensions, and in tissue sections.

% cells positive for lysozyme	
Cell suspension	Tissue section
8	7
10	12
10	15
14	12
11	16
13	19
21	15
24	21
20	17
8	12
15	29

demonstrate lysozyme in blood monocytes and nodal macrophages from tumour patients and control patients, and although such cells do not pose the same problems of identification as do tumour derived macrophages, the same rosette markers have been used to allow study of comparable cell populations.

One clear finding is the occurrence of strikingly higher percentages of lysozyme-positive monocytes in the blood of cancer patients when compared to the controls. This observation finds a precedent in the work of Rhodes, which has demonstrated increased activation of monocytes from cancer patients in terms of Fc receptor expression[111,112,138]. The possibility of a link between Fc receptor expression and lysozyme content is explored in a later section of this thesis.

Serum lysozyme concentration is known to be raised in patients with neoplastic disease[224,226,240], and the present observation of enhanced lysozyme activity in the monocytes of such patients offers a possible explanation as to the source of the enzyme. Currie, on the other hand, has suggested that adherent cells from the actual tumours may constitute the major source of lysozyme in both animals and humans[225,240]. However, this theory is based on lysozyme production by cells which had been separated from the tumour environment (q.v. pages 40 - 41), and the present study indicates that lysozyme can be detected in only a very small proportion of tumour-infiltrating macrophages. It is possible that tumour cells may depress the macrophages' ability to produce lysozyme.

Another interesting aspect of this finding of depressed lysozyme content in tumour-derived macrophages is its implication for macrophage activation. As discussed in the literature review, lysozyme content tends to mirror activation level (q.v. pages 39 - 40), and thus the present study would suggest that tumour-infiltrating macrophages are in a very low state of activation. However, many studies have shown that non-specifically cytotoxic (and hence activated) macrophages can be isolated from a variety of animal tumours[4,5,74,76,82], and this would seem to contradict the view expressed above. Nevertheless, it must be appreciated that all tests of cytotoxicity are carried out away from the tumour environment, and the lysozyme content of macrophages still in close proximity to tumour cells may be a more accurate reflection of the in vivo situation. In addition, there is good evidence that lysozyme may act as an effector substance in damaging tumour cells[246], and this would suggest that lysozyme deficiency in tumour-infiltrating macrophages is likely to be a major factor in the tumour's ability to escape destruction by the host.

Finally, the lysozyme content of macrophages from lymph nodes draining tumours does not appear to be markedly different from that of macrophages from nodes which are not draining tumours. It is of interest to note, however, that nodal macrophages are considerably less likely to contain lysozyme than monocytes, and that macrophages from nodes draining tumours are slightly more likely to contain the enzyme than are tumour infiltrating macrophages.

In conclusion, therefore, it would seem that peripheral blood monocytes from cancer patients are activated in terms of lysozyme content, but this does not appear to hold for macrophages from the actual tumours or from the regional nodes. However, it remains unclear whether the low level of lysozyme activity in tumour-infiltrating macrophages represents selective migration or tumour-induced depression.

5. Alpha-1-antitrypsin content of breast cancer associated macrophages

Introduction

Alpha-1-antitrypsin (A1AT) is a protease inhibitor with activity against a wide range of enzymes which is present in macrophages, and which is synthesised by them (q.v. pages 42 - 43). Its physiological role is unknown, and any part it might play in anti-tumour host defense is equally obscure. However, as it is such a ubiquitous component of macrophages, and because it is detectable both in tumours and in the serum of tumour-bearing patients, an investigation has been carried out into the distribution of A1AT in breast cancer-associated macrophages. Again, blood monocytes, macrophages from lymph nodes and tumour-infiltrating macrophages have been studied.

Method

a) Patients

Venous blood and primary tumours were obtained from 16 patients with breast cancer clinically confined to the breast and the axilla. From 12 of these patients, axillary lymph nodes, which were subsequently confirmed to be free of tumour by histological examination, were also available. Venous blood was obtained from 14 female control patients who were about to undergo surgery for non-neoplastic, non-inflammatory conditions, and lymph nodes were obtained from various sites in 10 similar patients during surgery. Details of these patients are given in the appendix.

b) Preparation of cells

Blood mononuclear cells were isolated using a Ficoll-Hypaque gradient, and cell suspensions from lymph nodes and tumours were prepared by collagenase digestion (q.v. pages 60 - 65).

c) Macrophage surface markers

Macrophage surface markers were determined by rosetting assays as in the previous study (q.v. page 102). To recap briefly, these consisted of Fc receptors, macrophage-associated antigen detected by the My6C3 monoclonal antibody, and Ia surface antigen detected by the BT2/9 monoclonal antibody. The controls were identical to those used in the previous study.

d) A1AT assay

A1AT was detected on cytocentrifuge preparations (q.v. pages 75 - 76) by means of the PAP immunoperoxidase method (q.v. pages 78 - 83), and the percentage of rosetting cells containing A1AT was estimated by counting 200 cells. The antiserum used was rabbit anti-human A1AT immunoglobulin fraction (DAKO A012) diluted 1 in 200 with 5 mls. of Tris-buffered saline containing 2% normal swine serum. As a control, a similar amount of test antiserum was incubated with 100µg. of human A1AT (Sigma) for 1 hour at room temperature before use. This procedure consistently abolished the immunoperoxidase staining. Positive controls consisting of a series of cyto-

centrifuge preparations of blood monocytes from one donation were used, and similar percentages of A1AT-positive cells were observed in each immunoperoxidase run.

e) Tissue sections

As in the previous study, formalin-fixed tissue sections of tumour and lymph node were also stained by the immunoperoxidase method for A1AT, after trypsin treatment.

f) Prognostic factors

The percentages of monocytes, nodal macrophages and tumour-infiltrating macrophages containing A1AT were compared to the four prognostic factors of lymph node status, oestrogen receptor status, tumour size and tumour grade (q.v. pages 58 - 59). This was done to find out whether any relationship existed between A1AT content and prognosis in the cancer patients.

Results

As the appearances of the cytocentrifuge preparations stained for A1AT were indistinguishable from those produced by the lysozyme assay, the reader is referred to pages 105 - 108 of the previous section. The overall results are shown in Figures 36 - 38, and can be summarised as follows:

a) Blood monocytes

Monocytes from the cancer patients and from the control patients contained A1AT to a similar extent ($P > 0.1$ by Wilcoxon's rank sum test for each marker).

b) Nodal macrophages

Macrophages from the tumour-draining nodes and macrophages from the control nodes were also similar in terms of A1AT content ($P > 0.1$ by Wilcoxon's rank sum test for each marker). However, as with lysozyme, A1AT was found significantly less frequently in tumour or control nodal macrophages than in tumour or control blood monocytes respectively ($P < 0.01$ by Wilcoxon's rank sum test for each marker).

c) Tumour-infiltrating macrophages

The tumour-infiltrating macrophages contained A1AT in significantly fewer numbers than did the blood monocytes from the cancer patients ($P < 0.001$ by Wilcoxon's rank sum test for the Fc receptor and My6C3 markers, and $P < 0.01$ for the Ia marker). There was no difference, however, between tumour-infiltrating macrophages and macrophages from the tumour-draining nodes, even when the data was paired ($P > 0.1$ by Wilcoxon's signed rank test for each marker).

d) Tissue sections

A comparison between the percentages of A1AT positive cells in non-rosetted cytocentrifuge preparations of tumours and nodes, and the percentage of A1AT positive cells on sections of these tissues is shown in tables 4 and 5. As with the lysozyme studies, the figures are reasonably comparable, suggesting that collagenase disaggregation of tumours and nodes does not substantially alter the percentage of cells containing A1AT.

Figure 36 Percentages of Fc receptor positive cells of the monocyte/macrophage series containing A1AT.

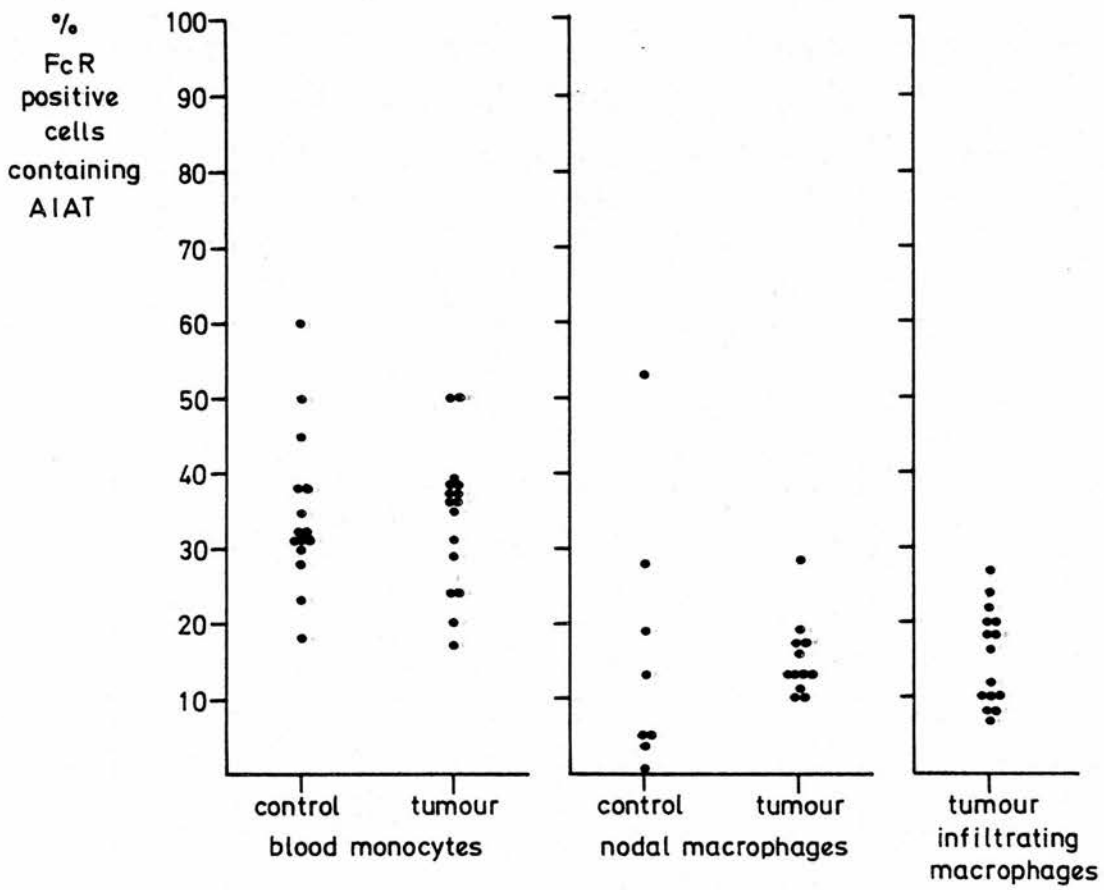


Figure 37 Percentages of My6C3 positive cells of the monocyte/macrophage series containing A1AT.

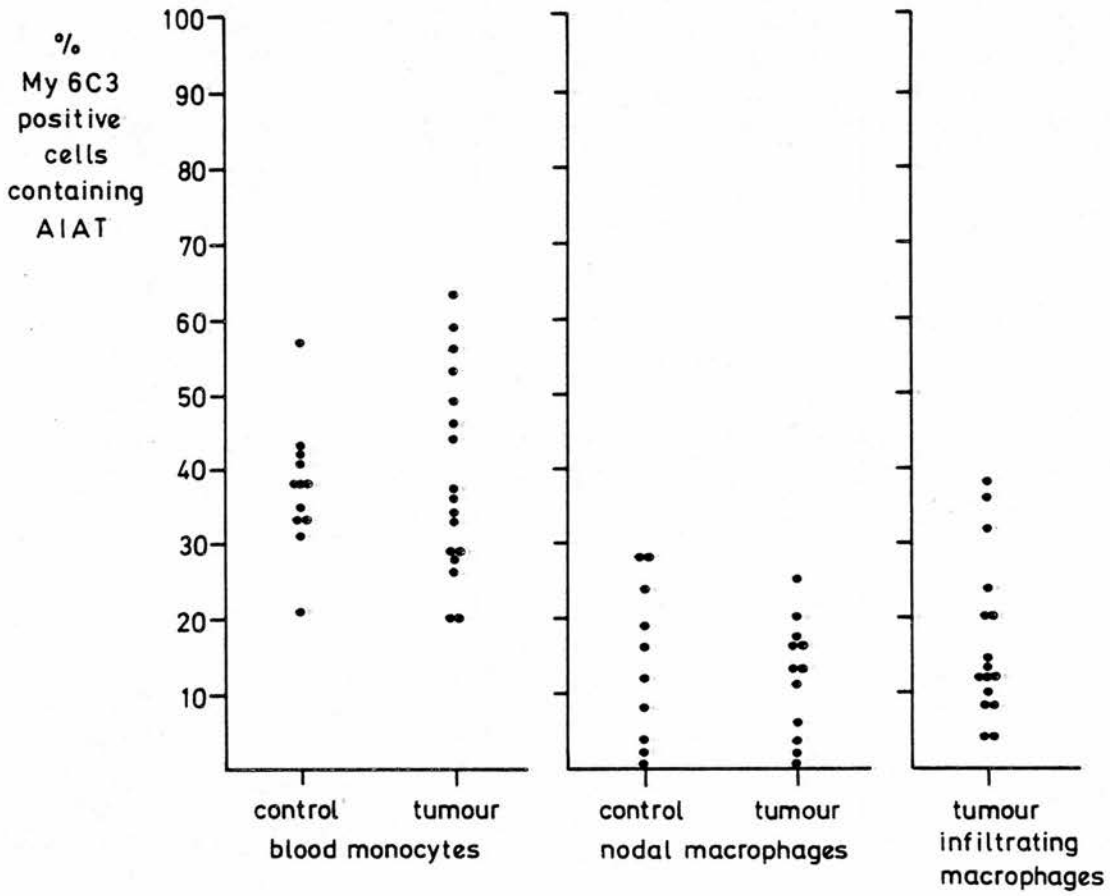


Figure 38 Percentages of Ia positive cells of the monocyte/macrophage series containing A1AT.

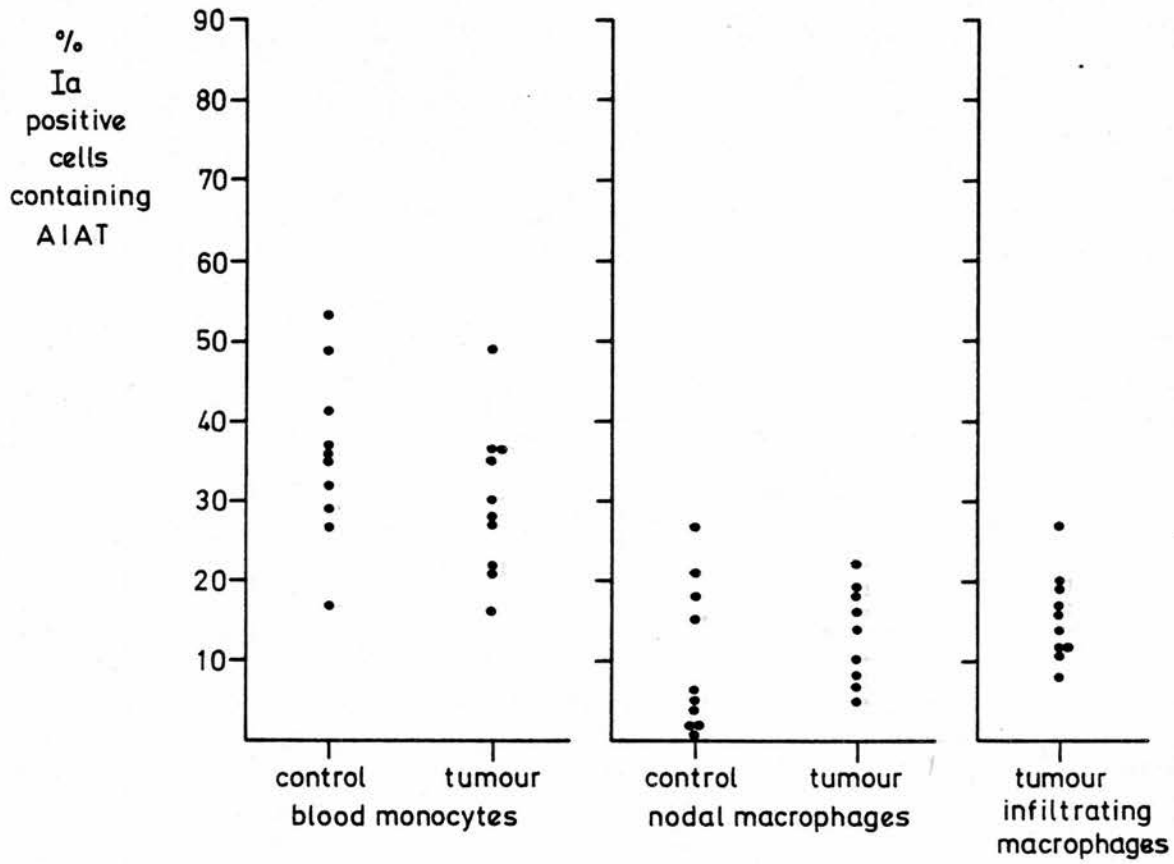


Table 4 Comparison of the percentages of the TOTAL CELL POPULATION containing A1AT in cytocentrifuge preparations from tumour cell suspension, and in tissue sections.

% cells positive for A1AT	
Cell suspension	Tissue section
16	5
6	<1
2	<1
7	9
9	8
5	3
16	21
8	1
12	18
8	<1
4	<1
10	3
7	<1

Table 5 Comparison of the percentages of the TOTAL NON LYMPHOCYTIC CELL POPULATIONS containing A1AT in cytocentrifuge preparations from lymph node cell suspensions, and in tissue sections.

% cells positive for A1AT	
Cell suspension	Tissue section
16	12
4	9
3	10
8	17
11	8
17	22
24	16
20	15
16	5
19	13
11	18

However, the immunostained tissue sections revealed that the distribution of A1AT within the breast tumours was very different from that of lysozyme. Although A1AT was located in a proportion of cells, the vast majority was to be found in the stroma surrounding the tumour cells (Figures 39 and 40), suggesting that the substance might be secreted or sequestered by the tumour. This pattern was a consistent finding in all 13 tumours studied in this way.

e) Prognostic factors

No relationship between macrophage A1AT content and any of the prognostic factors examined was found to exist.

Discussion

As with lysozyme, A1AT is found less frequently in tumour-infiltrating macrophages than in the monocytes of cancer patients, and lymph node macrophages are similarly deficient whether or not they are draining tumours. This is perhaps not surprising if, as suggested in the literature review, A1AT is an important regulator of macrophage enzymatic activity (q.v. pages 43 - 44). If this is so, a control mechanism to alter intracellular A1AT levels in parallel with levels of proteolytic enzymes will probably operate, and thus A1AT might be expected to fluctuate in concert with lysozyme. The difference between monocytes from cancer patients and monocytes from control patients which was observed with lysozyme was not seen in the case of A1AT, however, and it is therefore likely that only

Figure 39 Tissue section of invasive breast cancer immunostained for A1AT (red), and counter stained with haematoxylin (blue).

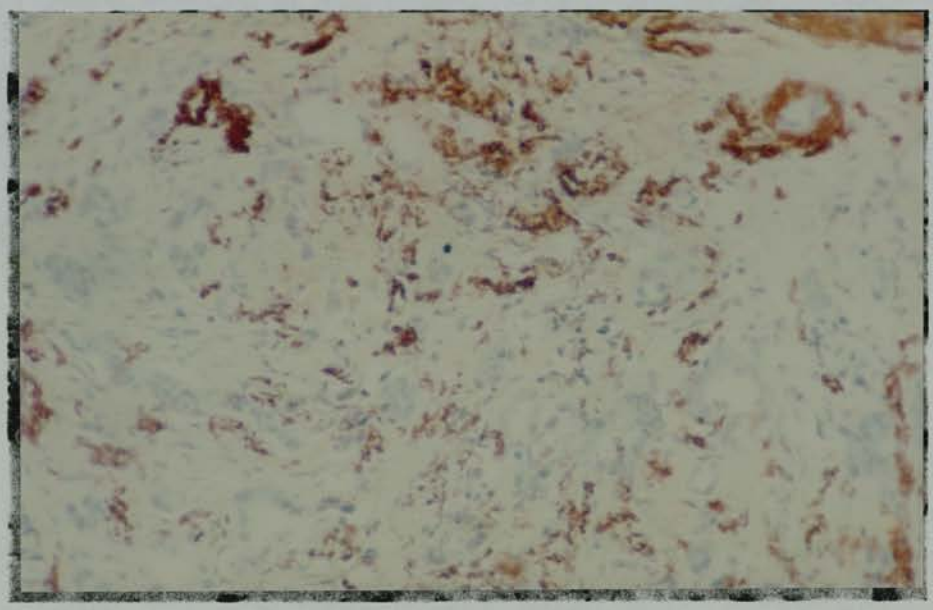
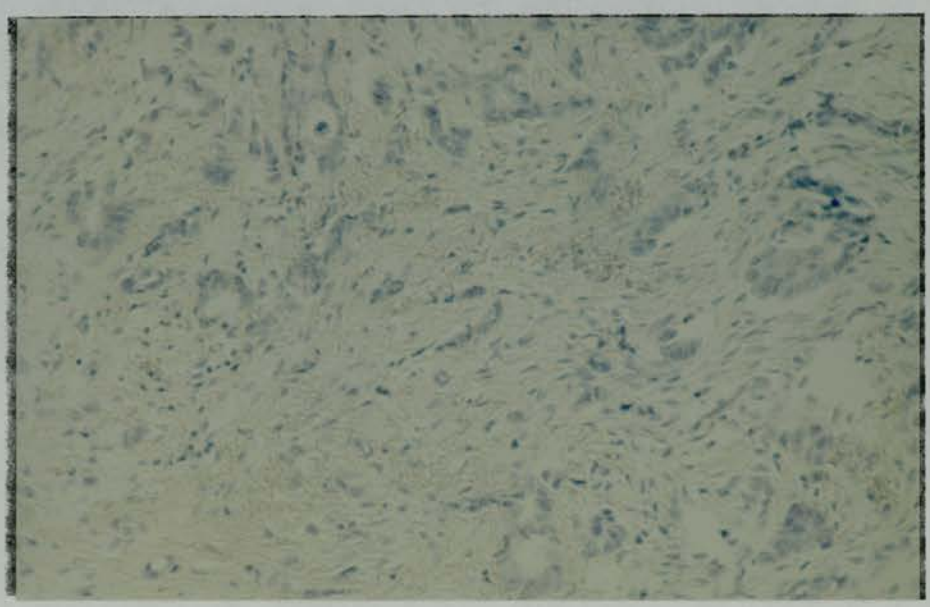


Figure 40 Adjacent tissue section with A1AT staining blocked by incubation of A1AT antiserum with purified A1AT.



gross changes in enzyme levels are accompanied by changes in A1AT levels.

The distribution of A1AT in breast tumours, as revealed by the tissue sections described in this study, is another aspect worthy of mention. The large extracellular deposits of A1AT suggest that substantial quantities are being produced or secreted by the tumour. The cell suspension studies would indicate, however, that, if A1AT is produced by the tumour, it must come from macrophages rather than from neoplastic cells, as cells lacking macrophage markers virtually never contained A1AT. Nevertheless, large amounts of A1AT within a tumour may well have an important influence on tumour behaviour, and the possibility exists that A1AT may provide protection against host-derived proteolytic enzymes.

6. The surface characteristics of breast cancer-associated macrophages

Introduction

In the preceding studies, the Fc receptor and the Ia surface antigen have been used as markers to identify macrophages. However, Fc receptor expression by macrophages can be heterogenous[11,12], and the same may be true of the Ia antigen. It was therefore decided to investigate the Fc receptor and the Ia antigen in their own rights, and to attempt to quantify their expression on blood monocytes and macrophages from lymph nodes and breast tumours. In addition, the C3 receptor, which was not useful as a marker because of its susceptibility to collagenase, has been studied.

Method

a) Patients

As in the previous studies, venous blood and tumours were obtained from 16 patients with breast cancer clinically confined to the breast and the axilla, and from 12 of these, tumour-free axillary lymph nodes were available. Fourteen venous blood samples and 10 lymph nodes were obtained in control patients undergoing surgery for non-neoplastic, non-inflammatory disease (see appendix). Blood monocytes were isolated on a Ficoll-Hypaque gradient, and macrophages from tumours and nodes were obtained by collagenase digestion (q.v. pages 60 - 65).

b) Fc receptors

Fc receptors on the cell surface were detected by EA rosette formation (q.v. pages 66 - 67). Cytocentrifuge preparations of the rosetted cell suspensions were made and stained with haematoxylin; the percentage of blood monocytes or nodal macrophage-like cells forming EA rosettes was then estimated by counting 200 cells. This was not possible in tumour cell preparations, however, as morphological identification of macrophages was not possible. Therefore, the number of erythrocytes forming each rosette in the tumour cell preparation was recorded, and a mean value from 100 rosettes in each sample was calculated. This was also done for the blood monocytes and nodal macrophages.

c) C3 receptors

C3 receptors on the cell surface were detected by EAC rosette formation, and controls consisted of erythrocytes coated with IgM alone (q.v. page 68). Cytocentrifuge preparations of the rosetted cells were made, and, as for the Fc receptor assay, percentages of rosetting monocytes and nodal macrophages were counted, and the mean number of erythrocytes per rosette calculated for monocytes, nodal macrophages and tumour-infiltrating macrophages.

d) The Ia antigen

Ia antigen on the cell surface was detected by rosette formation with sheep erythrocytes coupled to the BT2/9 (anti-Ia) mouse monoclonal antibody, and controls consisted of erythrocytes coupled to normal mouse immunoglobulin (q.v. pages 69 - 72). Again, percentages of rosetting monocytes and nodal macrophages were counted, and the mean number of erythrocytes per rosette calculated for monocytes, nodal macrophages and tumour-infiltrating macrophages.

e) Prognostic factors

The results of these three rosetting assays were then compared to the four prognostic factors of lymph node status, oestrogen receptor status, tumour size and tumour grade (q.v. pages 58 - 59), to ascertain whether a relationship might exist.

Results

The results are displayed in Figures 41 - 46, and can be summarised as follows:

a) Fc receptor expression (Figures 41 and 42)

Monocytes from the cancer patients formed rosettes significantly more frequently than did monocytes from the control patients ($P < 0.001$ by Wilcoxon's rank sum test), and macrophages from tumour-draining nodes formed rosettes significantly more often than macrophages from the control

Figure 41 The percentage of cells of the monocyte/macrophage series bearing Fc receptors by the EA rosetting technique.

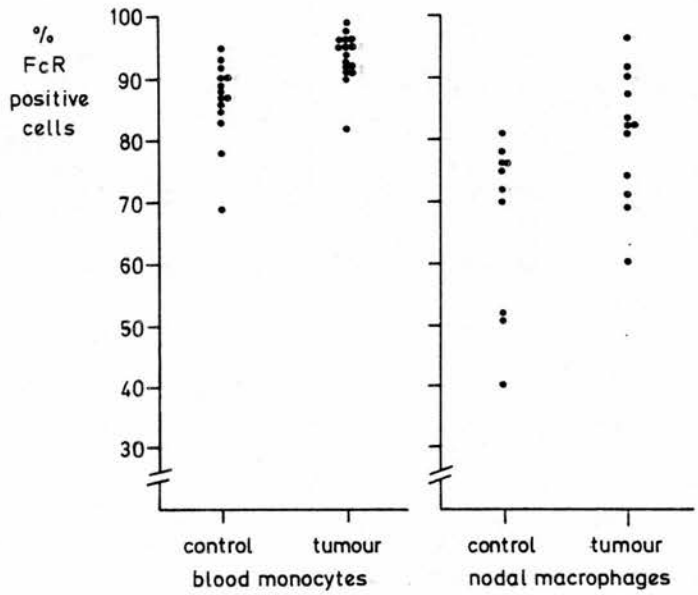
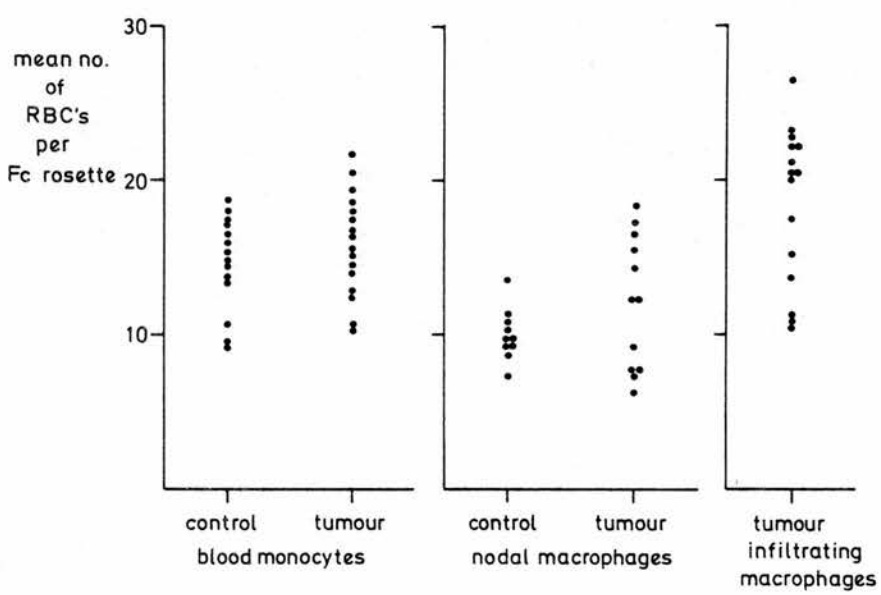


Figure 42 The mean number of erythrocytes forming EA rosettes on Fc receptor-positive cells of the monocyte/ macrophage series.



nodes ($P < 0.05$ by Wilcoxon's rank sum test). Nodal macrophages formed rosettes less frequently than blood monocytes both in the tumour and in the control situations ($P < 0.001$ by Wilcoxon's rank sum test).

When the mean rosette size was examined, again by Wilcoxon's rank sum test, nodal macrophages were found to have fewer erythrocytes per rosette than blood monocytes ($P < 0.05$ for the tumour samples, and $P < 0.01$ for the control samples), and tumour-infiltrating macrophages had more erythrocytes per rosette than the nodal macrophages ($P < 0.01$). When the tumour blood monocytes and tumour-infiltrating macrophages were paired, the latter had significantly higher mean values ($P < 0.05$ by Wilcoxon's signed rank test).

b) C3 receptor expression (Figures 43 and 44)

Because of the striking effect of collagenase on C3 receptor expression (q.v. pages 86 and 88), comparisons between samples which have been treated by the enzyme and those which have not cannot be performed. It is therefore only possible to compare monocytes with monocytes, and nodal macrophages with nodal macrophages or tumour-infiltrating macrophages.

There was no significant difference between tumour patients and control patients in terms of the percentages of rosette-forming monocytes or nodal macrophages ($P > 0.1$ by Wilcoxon's rank sum test). Similarly, no significant differences between the mean rosette sizes in tumour and control monocytes or nodal macrophages could be detected ($P > 0.1$ by Wilcoxon's

Figure 43 The percentage of cells of the monocyte/macrophage series bearing C3 receptors by the EAC rosetting technique.

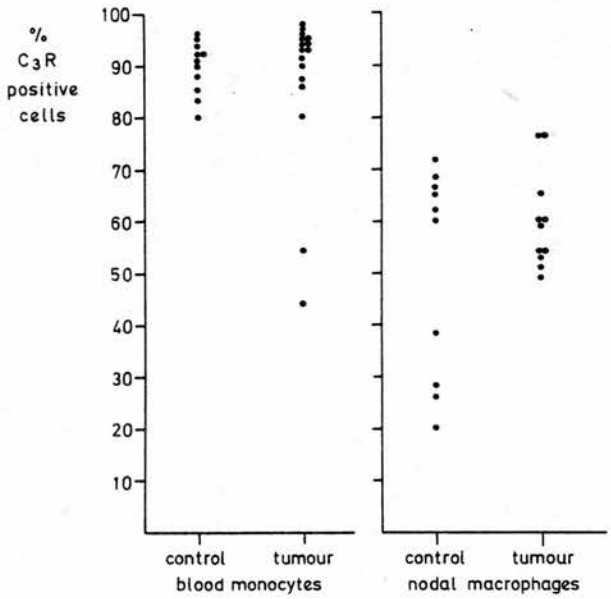
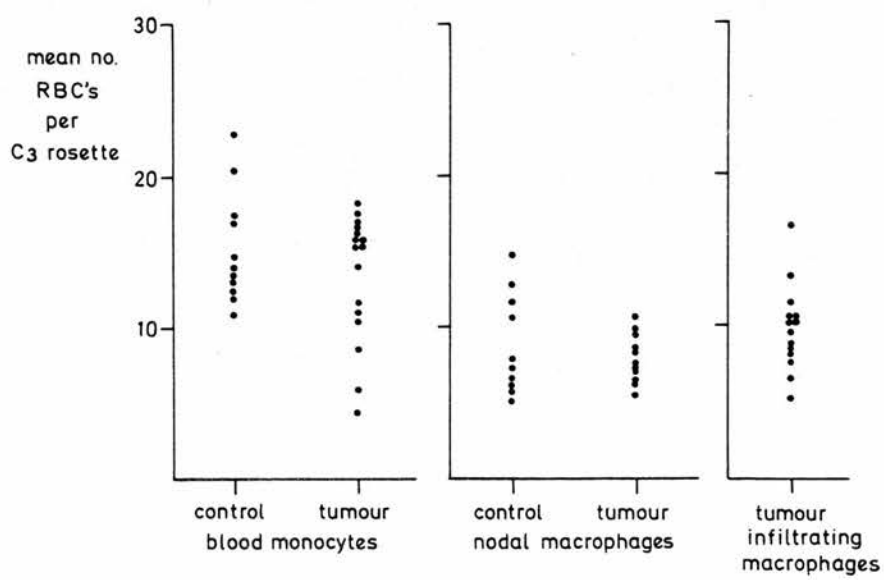


Figure 44 The mean number of erythrocytes forming EAC rosettes on C3 receptor-positive cells of the monocyte/macrophage series.



rank sum test). However, when paired, tumour-infiltrating macrophages showed significantly higher values than the corresponding nodal macrophages ($P < 0.01$ by Wilcoxon's signed rank test).

c) Ia antigen expression (Figures 45 and 46)

Blood monocytes and nodal macrophages from both tumour patients and control patients displayed a consistently high rate of rosette formation, and no significant differences were seen ($P > 0.1$ by Wilcoxon's rank test). Likewise, the mean rosette sizes were similar in monocytes and nodal macrophages from tumour and control patients, and in tumour-infiltrating macrophages ($P > 0.1$ by Wilcoxon's rank sum test or Wilcoxon's signed rank test for paired data).

d) Prognostic factors

No relationship between the expression of Fc receptors, C3 receptors or Ia antigen on monocytes, nodal macrophages or tumour-infiltrating macrophages and the four prognostic factors in the tumour patients could be detected.

Discussion

In this study, Fc receptor expression appears to be enhanced in blood monocytes and nodal macrophages from breast cancer patients. This finding is in accord with the work of Rhodes, who has shown that monocytes from patients with lung cancer and other tumours have increased affinity for the Fc portion of IgG[111,112,138]. However, Rhodes also demonstrated

Figure 45 The percentage of cells of the monocyte/macrophage series bearing Ia antigen by the rosetting technique.

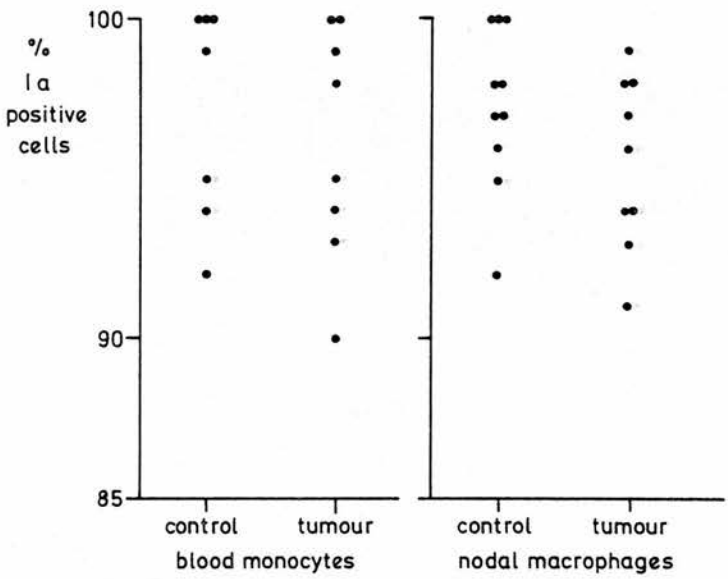
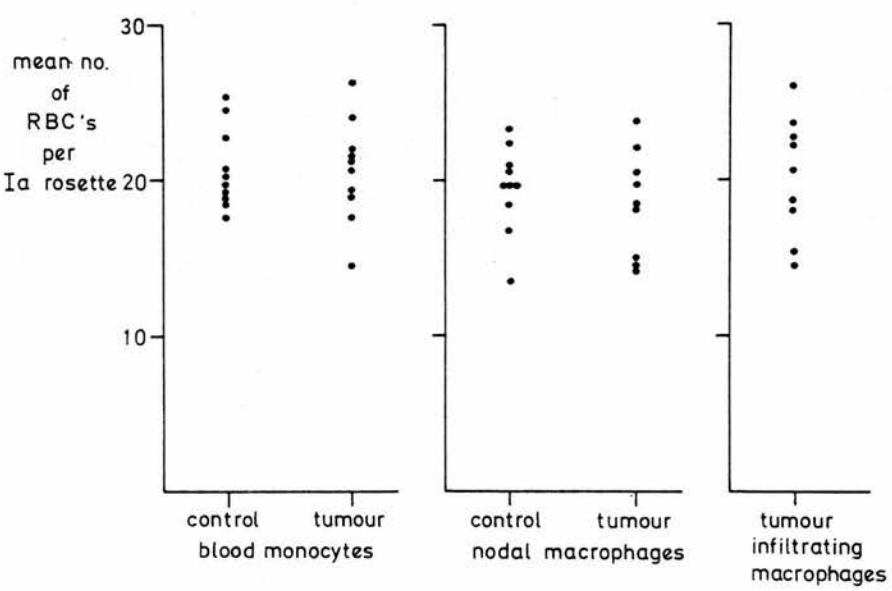


Figure 46 The mean number of erythrocytes forming Ia antigen-detecting rosettes on Ia antigen-positive cells of the monocyte/macrophage series.



that pulmonary alveolar macrophages from the vicinity of lung tumours had decreased Fc receptor activity[111], a finding which is somewhat at variance with the present work as the Fc receptor positive cells from the breast tumours formed rosettes of greater magnitude than did the corresponding blood monocytes.

This latter observation suggests that macrophages from breast tumours might in fact have enhanced Fc receptor activity, but one or two points must be made before reaching this conclusion. Firstly, a slight increase in Fc receptor expression occurs with collagenase treatment (q.v. pages 86 and 88), and thus the preparation of the tumour cell suspension might enhance this parameter. Secondly, tumour-associated macrophages are larger than monocytes, and the bigger rosettes might merely reflect increased surface area. On the other hand, the tumour-infiltrating macrophages also showed increased Fc receptor activity over the nodal macrophages, which are of similar size and which have been exposed to collagenase. It would seem reasonable, therefore, to conclude that tumour-infiltrating macrophages which do have Fc receptors express them at least as strongly as monocytes and nodal macrophages, although the possibility remains that a subgroup which are entirely devoid of these receptors might exist.

It is also of interest to note that nodal macrophages have diminished Fc receptor activity compared to monocytes, but this holds for all the patients studied, and cannot be ascribed to the tumour-bearing state.

Turning to the C3 receptor, there are no reports which have examined C3 receptor heterogeneity in macrophages

associated with cancer in humans. In the present study, comparisons are hampered by the effect of collagenase (Sigma, type I) on C3 receptor expression, but tumour-infiltrating macrophages do seem to have a greater affinity for C3 indicators than nodal macrophages, although a differential effect of collagenase on nodes and tumours cannot be ruled out. Certainly, there is no difference in C3 receptor expression between the blood monocytes of cancer patients and those of control patients.

Finally, in the case of Ia antigen expression, no heterogeneity has been detected. All the macrophages studied - from blood and nodes from tumour patients or control patients and from breast carcinomas - displayed uniformly high levels of Ia antigen expression.

In conclusion, this study has confirmed previous observations that monocytes from cancer patients have elevated Fc receptor activity, and it has shown that the same holds for macrophages isolated from tumour-draining lymph nodes. The suggestion that macrophages from the actual tumours have diminished Fc receptor activity has not, however, been upheld. Similar variation in the C3 receptor activity or Ia antigen expression of monocytes and nodal macrophages was not seen, however, although there is evidence that, despite collagenase treatment, tumour-infiltrating macrophages do bear C3 receptors to a significant extent.

7. Phagocytosis in breast cancer-associated macrophages

Introduction

In the literature review, it was pointed out that, although phagocytic mechanisms are unlikely to play a part in tumour cell killing, the ability of macrophages to ingest various particles may reflect level of activation and hence anti-tumour capacity (q.v. pages 34 - 36). In the present study, therefore, the ability of monocytes and macrophages from breast cancer patients to phagocytose IgG-coated erythrocytes has been assessed, and compared to the phagocytic capacity of monocytes and nodal macrophages from control patients.

Method

a) Patients

Venous blood, tumours and axillary lymph nodes were obtained from 10 patients with breast cancer clinically confined to the breast and axilla. Venous blood was also obtained from 10 female control patients who were about to undergo surgery for non-neoplastic, non-inflammatory conditions, and lymph nodes were obtained from various sites in 10 similar patients during surgery. Details of these patients are given in the appendix.

b) Preparation of cells

Blood mononuclear cells were isolated using a Ficoll-Hypaque gradient, and cell suspensions from lymph nodes and tumours were prepared by collagenase digestion (q.v. pages 60 - 65).

c) Phagocytosis assay

Cell suspensions from blood, lymph nodes and tumours were incubated with ox erythrocytes coated by rabbit IgG against ox erythrocytes for 30 minutes at 4°C, which allowed EA rosettes to form without significant phagocytosis of the red cells (q.v. pages 66 and 74). The rosetted mixtures were then incubated at 37°C for exactly 2 hours, cytocentrifuge preparations were made, and the percentages of rosetting macrophages or monocytes which had phagocytosed at least one red cell were estimated by counting 200 cells in each instance. Counting was done using phase contrast microscopy, which allowed confirmation of ingestion of the red cells. In addition, when immunoperoxidase assays were done on these preparations, the erythrocytes forming the EA rosettes stained red, because the rabbit IgG coating them was picked up by the assay (q.v. pages 78 - 83). When an erythrocyte was phagocytosed, the red coloration disappeared (Figures 47 - 48), presumably because the surface immunoglobulin was not available to the assay, or because it had been removed by the ingestion process. This phenomenon provided another check for true phagocytosis.

Results

The overall results are given in Figure 49. No difference was observed between the phagocytic capacities of Fc receptor bearing monocytes from the cancer patients and the control patients ($P > 0.05$ by Wilcoxon's rank sum test);

Figure 47 An Fc receptor-positive blood monocyte which has phagocytosed an erythrocyte.

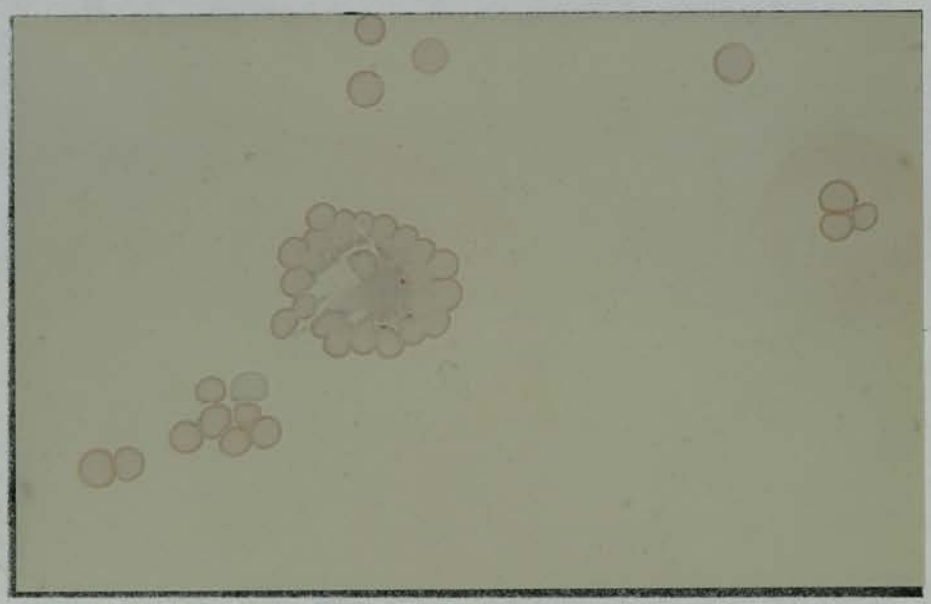


Figure 48 An Fc receptor-positive tumour macrophage which has phagocytosed several erythrocytes.

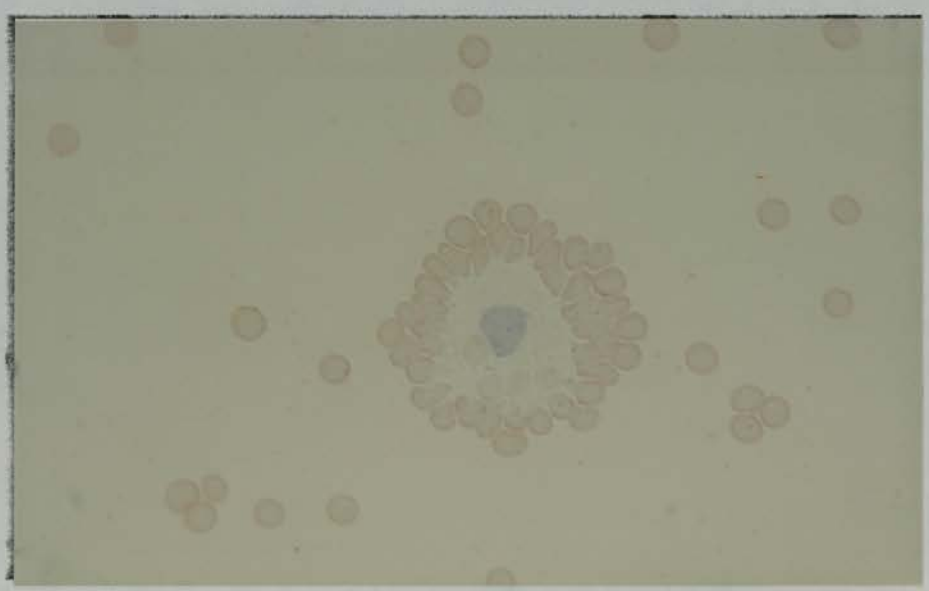
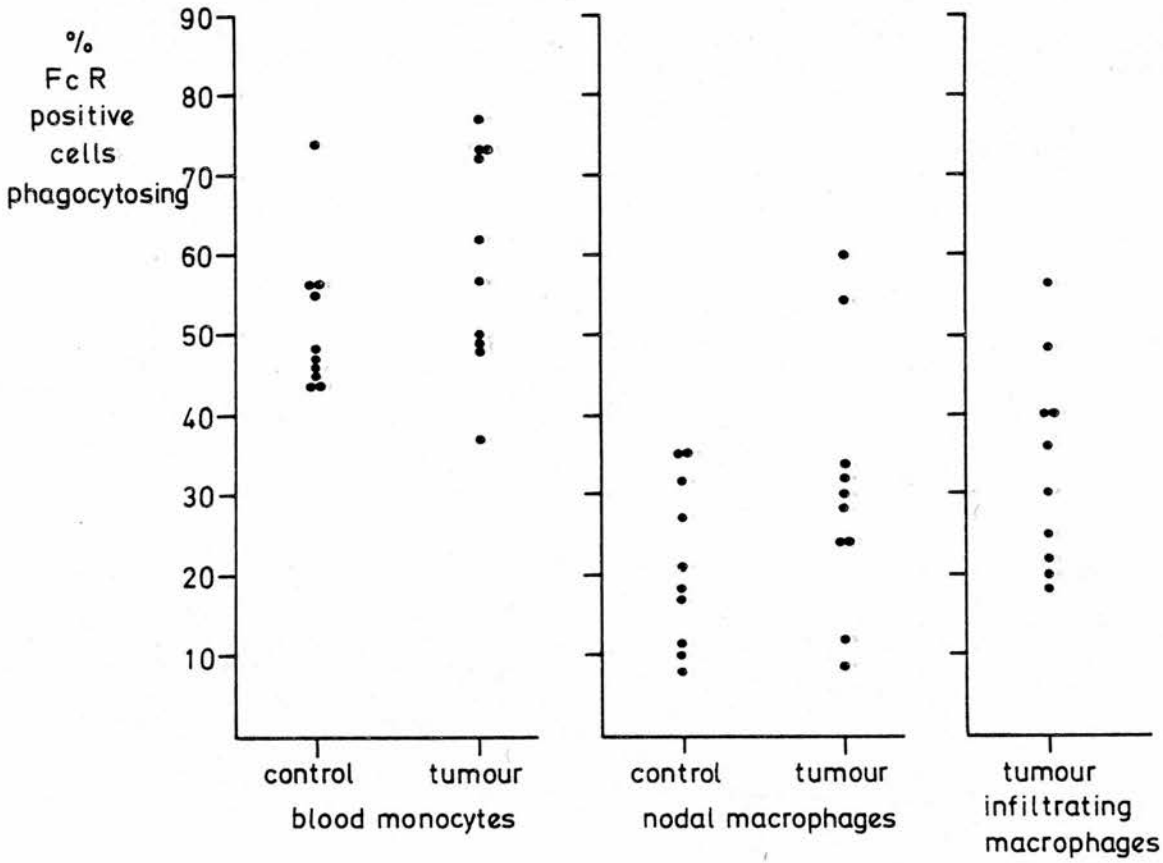


Figure 49 The percentages of Fc receptor-bearing cells which had phagocytosed at least one indicator erythrocyte after incubation at 37°C for 2 hours.



similarly, no difference was noted between macrophages from tumour-draining and control lymph nodes ($P > 0.1$ by Wilcoxon's rank sum test), and no difference was seen between nodal macrophages and tumour-infiltrating macrophages - whether or not they were paired ($P > 0.1$). However, the nodal macrophages from the tumour patients phagocytosed red cells significantly less frequently than did monocytes ($P < 0.01$), but this was not tumour-related as the same held true for the control patients ($P < 0.001$). Finally, the tumour-infiltrating macrophages phagocytosed erythrocytes significantly less frequently than did the monocytes from the tumour patients ($P < 0.01$ by Wilcoxon's rank sum test).

Discussion

It is important to realise that, in this study, only Fc receptor bearing macrophages and monocytes have been studied. Because of this, although the phagocytic mechanism which has been measured is mediated by IgG, variations in the presence or absence of Fc receptor expression should not alter the observed rate of phagocytosis. However, differences in the degree of receptor expression may have an effect, and it is important to look at the results from this point of view.

In the previous study, it was found that the degree of Fc receptor expression in nodal macrophages, as measured by mean rosette size, was significantly less than that of monocytes (Figure 42, page 132). This in itself might explain the present finding of depressed ingestion of IgG-coated

red cells by nodal macrophages. However, it was also shown in the previous study that tumour-infiltrating macrophages exhibited a greater mean rosette size than monocytes (Figure 42, page 132), and yet they display markedly less phagocytosis. It would therefore seem that IgG-mediated phagocytosis, as opposed to EA rosette formation, is impaired in macrophages from breast tumours, again suggesting depressed activation of these cells.

It is perhaps surprising that neither monocytes nor nodal macrophages from tumour patients displayed deficient phagocytosis, as the clearance of colloid from both the blood and regional nodes of cancer-bearing animals has been found to be depressed[100,102]. However, the assay used in this study was specific for phagocytosis initiated by interaction with the Fc fragment of IgG, and the mechanisms involved may be substantially different from those operating for colloid ingestion.

8. Characterisation of sinus histiocytosis in axillary lymph nodes draining breast cancer

Introduction

Sinus histiocytosis in lymph nodes draining breast cancer has long been associated with a favourable prognosis (for a detailed discussion, see Part I of this thesis, volume 1 pages 82 - 85). However, despite close attention from histopathologists, the nature of the sinus histiocyte remains obscure. During the course of the work detailed in this thesis, some light has been shed on this problem, and in the present section, it is proposed to examine the information which has arisen from the previous studies regarding the characteristics of this cell.

Throughout the previous studies, cell suspensions from lymph nodes have been made by collagenase digestion of nodal stroma to obtain large, non lymphoid cells (q.v. page 65). The assumption has been that these cells are macrophages, and the aim of this section is to assess how justified this assumption is, and to establish whether a relationship between histological sinus histiocytosis and these cells might exist.

Method

In all, 16 individual axillary lymph nodes draining breast tumours were studied, although every lymph node was not examined in each experiment. Cell suspensions from these nodes were prepared by collagenase digestion as previously described (q.v. page 65), and seven macrophage markers were

used to characterise the large, non-lymphoid cells. Rosetting reactions were used to identify Fc receptors, C3 receptors, the My6C3 macrophage-associated surface antigen, and the Ia surface antigen (q.v. pages 66 - 74). Phagocytosis of IgG-coated erythrocytes was then assessed by incubating Fc receptor-detecting rosettes at 37°C for 2 hours (q.v. page 140). Finally, immunoperoxidase staining was used to identify lysozyme and A1AT within the cells (q.v. pages 78 - 83).

To investigate the relationship between the large, non-lymphoid cells and histological sinus histiocytosis, the percentage of the total cell suspension comprising large cells was estimated by counting 200 cells on cytocentrifuge preparations, and compared with the percentage of the cross-sectional area of the lymph node occupied by sinus histiocytosis. This latter percentage was calculated by projecting a histological section of the lymph node in question on to a screen, and superimposing a fine grid onto the image by means of an overhead projector. By counting the number of squares overlying areas of sinus histiocytosis (Figure 50) it was possible to estimate the percentage of the node occupied by this feature.

Results

The percentages of large cells from lymph node cell suspensions displaying the various cell surface markers are given in the appendix and also shown in Figures 41 (page 132), 43 (page 134), and 45 (page 136). For convenience, these are summarised in table 6, which demonstrates that the vast

Figure 50 Sinus histiocytosis with superimposed grid.

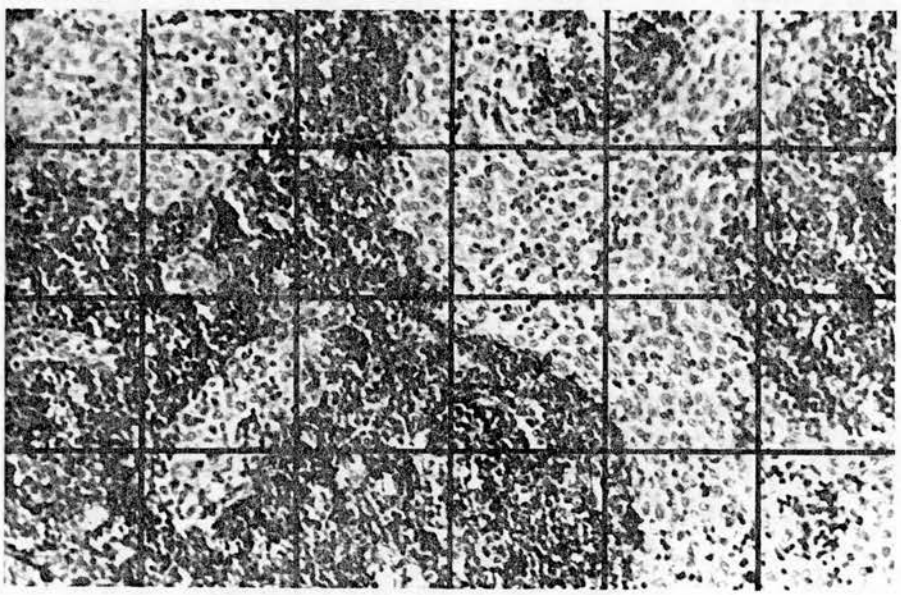


Table 6 Percentages of large, sinus histiocyte-like cells from tumour-draining lymph node cell suspensions showing various markers.

Marker	Mean %	Standard error	n
My6C3	94.8	0.6	12
Fc receptors	80.5	2.9	12
C3 receptors	59.7	2.8	11
Ia antigen	95.6	0.9	9
Phagocytosis by Fc receptor bearing cells	31.5	5.5	10
Lysozyme	14.2	1.7	12
A1AT	13.6	6.7	12

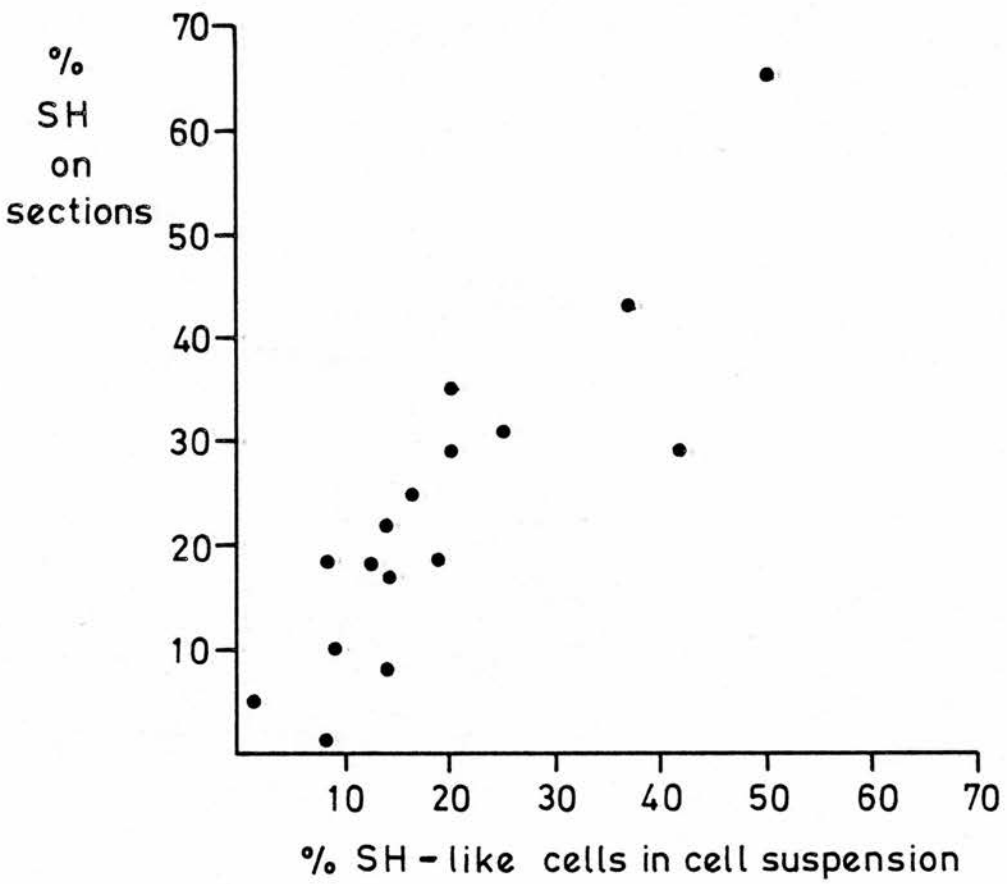
majority of these cells expressed the My6C3 antigen, Fc receptors and Ia antigens. Only about 60% had C3 receptors, but it must be remembered that collagenase reduces the expression of the C3 receptor (q.v. pages 83 - 91). Also in table 6, it is shown that about 30% of Fc receptor-positive large cells from tumour-draining nodes phagocytosed indicator erythrocytes under the specified conditions (see also Figure 49, page 142), and in the region of 15% contained lysozyme and A1AT by immunoperoxidase assay (see also table 2, page 114 and table 4, page 125).

Figure 51 illustrates the relationship between the percentage of large, sinus histiocyte-like cells seen on cytocentrifuge preparations of the lymph node cell suspensions, and the percentage cross-sectional area occupied by histological sinus histiocytosis. Clearly, there is a close correlation, and this is highly significant by Kendall's rank correlation test ($P < 0.001$).

Discussion

In this study, it has been demonstrated that the majority of large, sinus histiocyte-like cells obtained by collagenase disaggregation of the stroma from breast cancer-draining lymph nodes bear My6C3 non-specific macrophage-associated antigen, Fc receptors and Ia antigens. In addition, a substantial number have C3 receptors, despite the collagenase. This suggests that such cells are, in the main, macrophages, because, although these markers are not exclusive to macrophages, the combination of all four provides strong evidence to support this conclusion.

Figure 51 Correlation between percentage of sinus histiocyte-like cells in nodal cell suspensions and cross-sectional area of lymph node displaying histological sinus histiocytosis.



However, three other macrophage markers - phagocytosis, lysozyme and A1AT - were observed less frequently, which suggests that these nodal cells are not at a very high level of activation. The finding that breast biopsy can induce sinus histiocytosis to a certain extent (see part I of this thesis, volume 1, pages 184 - 188) would tend to support the view that this particular reactive change is unlikely to represent an active anti-tumour response.

Having established some of the characteristics of the large nodal cells, it remains to ask if these cells are indeed sinus histiocytes. The relationship between the incidence of these cells in cell suspension and the area occupied by sinus histiocytosis in the same nodes would suggest that they are, because, although the percentages naturally do not coincide, they correlate very closely.

In conclusion, sinus histiocytosis, whatever its prognostic implications might be, appears to represent an accummulation of macrophages within a node, albeit macrophages at a rather low level of activation.

9. Macrophage content and prognostic factors in human breast cancer

Introduction

Because macrophages are protective host cells, it has long been assumed that their presence in tumours must be beneficial. Early work suggested that the macrophage content of transplantable animal tumours positively correlated with immunogenicity and was inversely related to metastasising tendency[31,75,76], but more recent studies have refuted these initial conclusions[78-80].

However, the macrophage content of human tumours has been very little studied, and the effect of intratumoural macrophages on the behaviour of a single tumour type might not be mirrored by the different macrophage contents displayed by various experimental tumours. It was therefore decided to estimate macrophage content in a series of human breast cancers and to compare this to the incidence of established prognostic indices.

Method

a) Patients

40 patients with invasive breast cancer, clinically confined to the breast or the axilla were studied. Lymph node status, tumour size, tumour grade and oestrogen receptor status (q.v. pages 58 - 59) were obtained for each patient, if available.

b) Preparation of tumours

Cell suspensions from tumours were prepared by mechanical disaggregation and collagenase digestion, as previously described (q.v. pages 63 - 64).

c) Macrophage markers

Three separate rosetting reactions were used to identify macrophages in the tumour cell suspension - Fc receptor detection using ox erythrocytes coated with rabbit IgG against ox erythrocytes, identification of macrophage-associated antigen by My6C3 mouse monoclonal antibody coupled to sheep erythrocytes, and identification of Ia surface antigen by BT2/9 mouse monoclonal antibody, also coupled to sheep erythrocytes (q.v. pages 66 - 74). Cytocentrifuge preparations of these rosetted cell suspensions were then made, and the percentage of the total population comprising rosetted cells was estimated by counting 200 cells.

d) Statistical analysis

Because the numbers involved in these studies were greater than in previous studies, and because the groups of values were fairly evenly distributed, Student's t test was used to analyse the data.

Results

As can be seen from Figures 52 - 55, very little difference existed between the macrophage contents of tumours which were associated with the poor prognostic factors of

positive lymph node status, tumour size greater than 4.0 cm., histological grade III, or negative oestrogen receptor status, and the macrophage content of tumours which were not ($P > 0.1$ for each marker by Student's t test). However, when each poor prognostic factor (as defined above) was ascribed a value of 1, and the patients grouped according to whether they had a score of 0 - 2 or 3 - 4, a different picture was seen (Figure 56). In this case, the tumours associated with the poorer prognosis had significantly higher macrophage contents when compared to the tumours in the better prognostic group ($P < 0.01$ for the Fc receptor and My6C3 antigen markers, and $P < 0.05$ for the Ia antigen marker, by Student's t test).

Discussion

From this study, it appears that, far from being associated with a good prognosis, a high macrophage content tends to relate to factors indicating a poor prognosis in breast cancer. It is clear, however, that the present study does not exclude the possibility that macrophage content might constitute an independent prognostic variable, and only prolonged follow-up of the patients can settle this issue. However, as prognostic factors carrying the same implications tend to co-exist (see Part I of this thesis, volume 1 pages 24 - 36), it would seem unlikely that a high macrophage content will transpire to have a favourable influence.

Interestingly, some studies in mice have suggested that macrophages can stimulate tumour growth under certain

conditions (q.v. pages 14 - 15), and this lends further credence to the view that macrophages in a tumour may be detrimental. It is not yet possible, however, to be sure that the intratumoural macrophages in breast cancer are actively encouraging neoplastic growth, but it does seem unlikely that a high macrophage content can ever be regarded as a favourable prognostic factor in this tumour.

Figure 52 Relationship between tumour macrophage content, as estimated by three separate markers, and lymph node status.

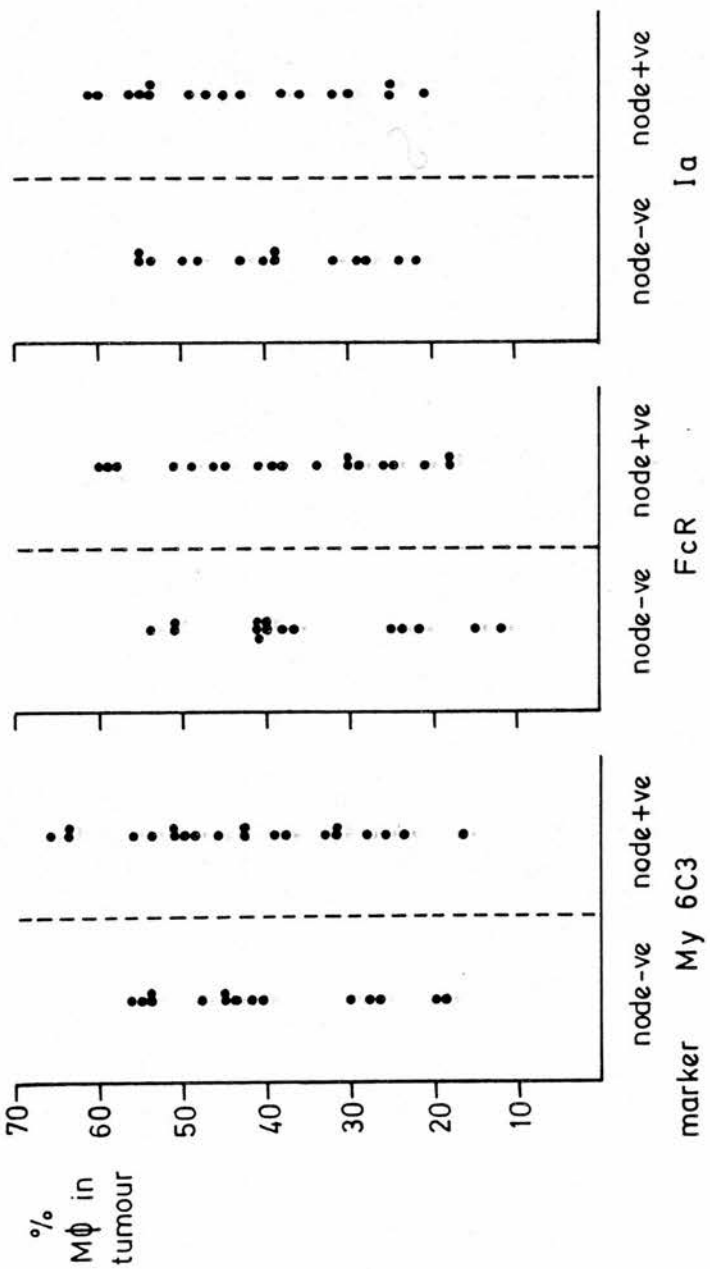


Figure 53 Relationship between tumour macrophage content, as estimated by three separate markers, and tumour size.

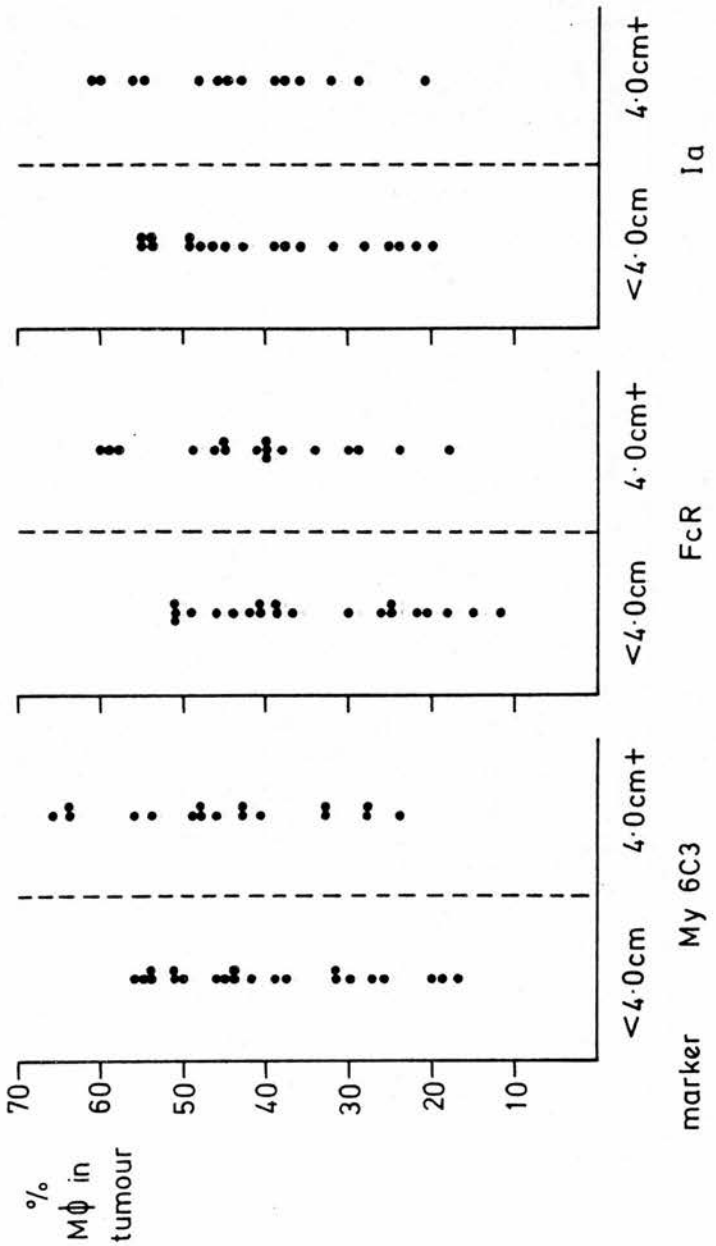


Figure 54 Relationship between tumour macrophage content, as estimated by three separate markers, and tumour grade.

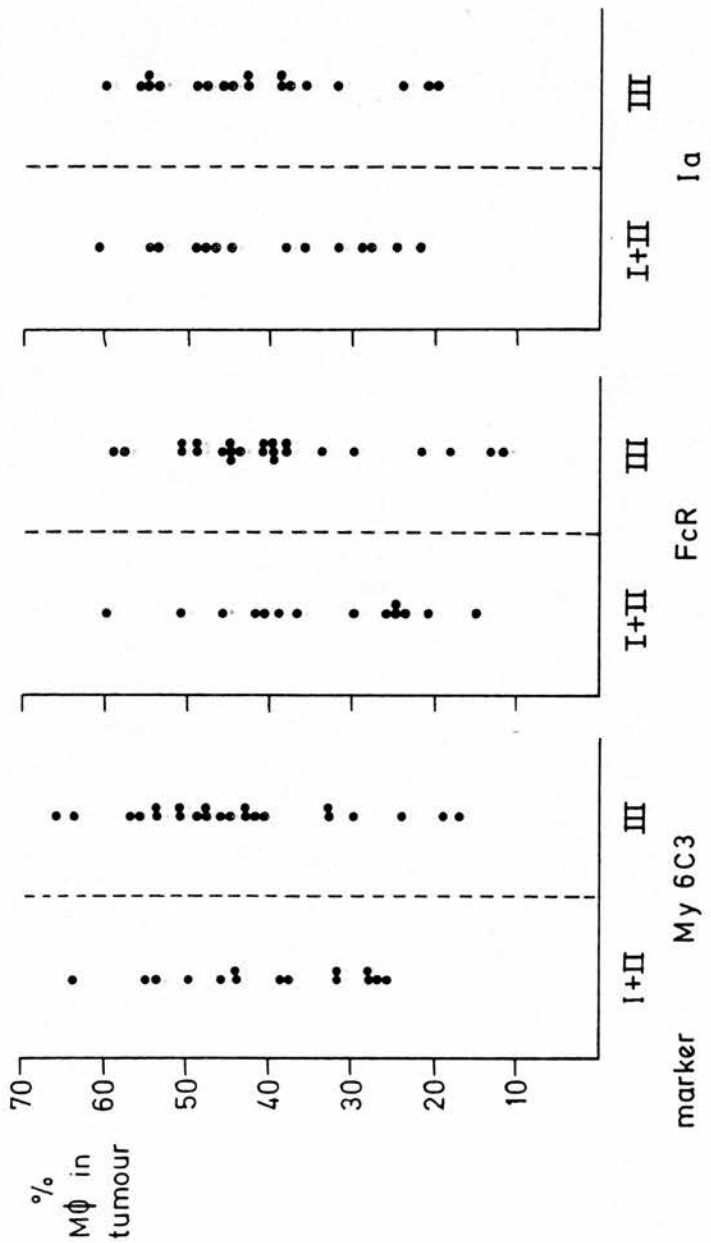


Figure 55 Relationship between tumour macrophage content, as estimated by three separate markers, and oestrogen receptor status.

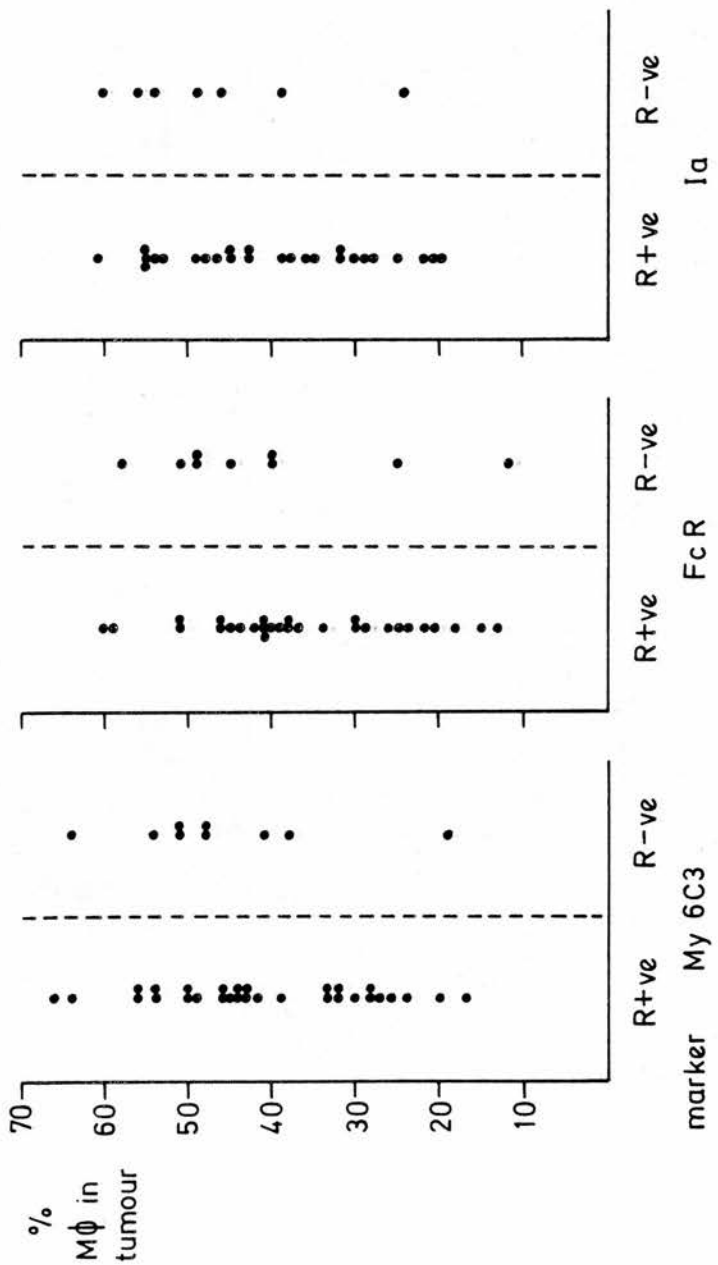
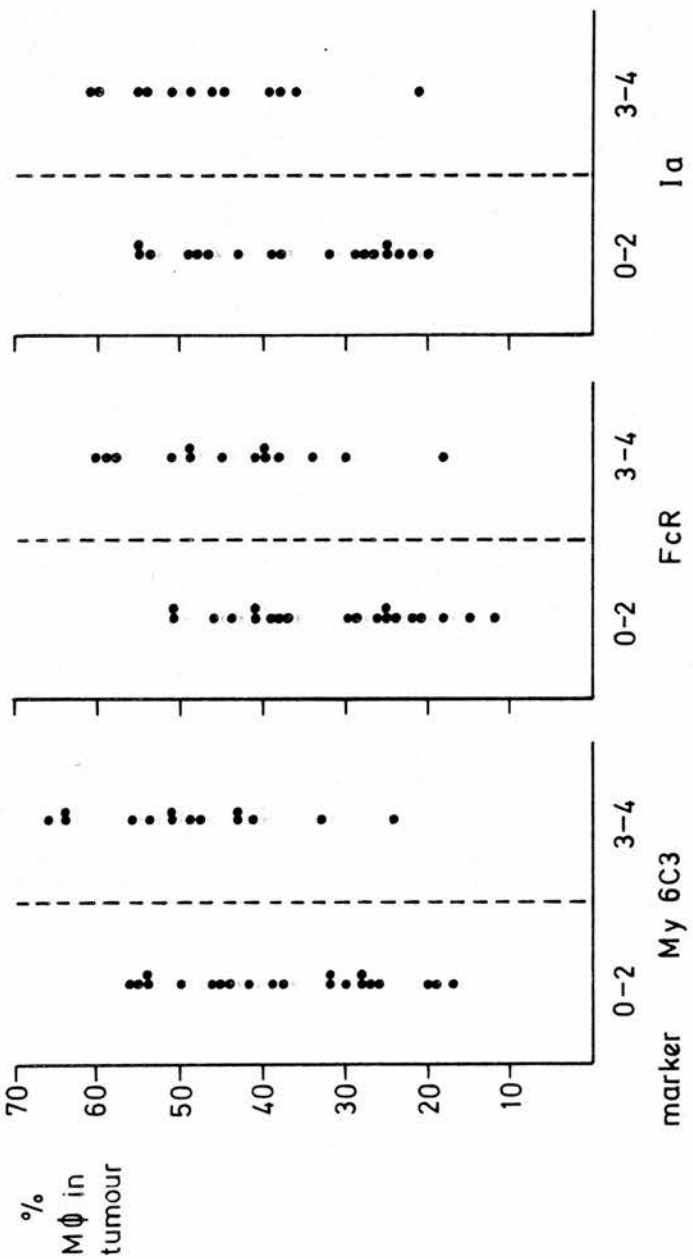


Figure 56 The relationship between tumour macrophage content, as estimated by three markers, and the number of poor prognostic factors.



10. Some observations on normal human monocytes

During the course of the experiments some incidental observations were made regarding the relationship between the Fc and the C3 receptor and lysozyme and A1AT content of monocytes. As a result of these observations, two studies were carried out on blood monocytes from the control patients, and these are described below.

10 i) The relationship between the expression of Fc receptors or C3 receptors and the presence of cytoplasmic lysozyme or A1AT in normal human monocytes

Introduction

The expression of Fc receptors on the cell surface has been taken as a measure of macrophage activation, largely because various activating agents can cause an increase in this parameter[136,173-175]. There is also reason to believe that lysozyme content is another measure of activation, as administration of activating substances which have anti-tumour effects in mice is accompanied by an increase in serum lysozyme levels[70,274].

The purpose of the present study was to establish whether a relationship between Fc receptor expression and lysozyme content in monocytes exists, as this would help to clarify whether or not lysozyme is an index of macrophage activation. In addition, the relationship between Fc receptor expression and A1AT content has been examined, and similar

studies involving the C3 receptor have been carried out.

Method

a) Patients

Venous blood samples were obtained from 14 patients who were about to undergo surgery for non-neoplastic, non-inflammatory conditions.

b) Preparation of cells

Blood mononuclear cells were isolated using a Ficoll-Hypaque gradient as previously described (q.v. pages 60 - 62).

c) Identification of surface receptors

Fc and C3 receptors on monocytes were detected by the rosetting techniques which are described in the general methods section (q.v. pages 66 - 68), and the appropriate controls were used.

d) Identification of lysozyme and A1AT

Lysozyme and A1AT were detected by the immuno-peroxidase assay as previously described (q.v. pages 78 - 83), and this was carried out on cytocentrifuge preparations of the rosetted monocytes. 100 monocytes from each immunostained cytocentrifuge preparation were then counted, and a note was taken of the proportion of receptor-bearing cells which were positive on immunoperoxidase staining. Similarly, the proportion of receptor-negative cells which were immunoperoxidase positive was recorded. In addition, the number of red cells forming

each rosette was counted, and a mean rosette size was calculated for immunoperoxidase positive and negative receptor-bearing monocytes in each sample.

Results

The results are shown in Figures 57 - 62, and they clearly demonstrate that the presence of both Fc receptors and C3 receptors correlates strongly with the presence of lysozyme and A1AT.

Virtually all of the lysozyme or A1AT positive monocytes had both types of receptor, and very few receptor-negative cells contained lysozyme or A1AT. These trends are displayed in Figures 57 - 60, and are all highly significant ($P < 0.001$) by Cox's procedure for combining several regressions which have a binary response[294].

When the mean rosette sizes for the receptor-bearing immunoperoxidase positive monocytes were compared to those for the receptor-bearing immunoperoxidase negative cells (Figures 61 - 62), both Fc and C3 receptor activity was significantly greater by this parameter in lysozyme positive cells ($P < 0.01$ in both cases, by Wilcoxon's rank sum test). Fc receptor activity was also greater in A1AT positive cells ($P < 0.01$), but this relationship was not found with the C3 receptor ($P > 0.1$).

Discussion

This study has indicated that, in normal blood monocytes, Fc and C3 receptor expression are both strongly

Figure 57 The percentages of Fc receptor-positive and Fc receptor-negative monocytes displaying lysozyme in 14 individual blood mononuclear cell preparations. The figures above each column indicate the actual number of cells counted (denominator) and the number positive for lysozyme (numerator).

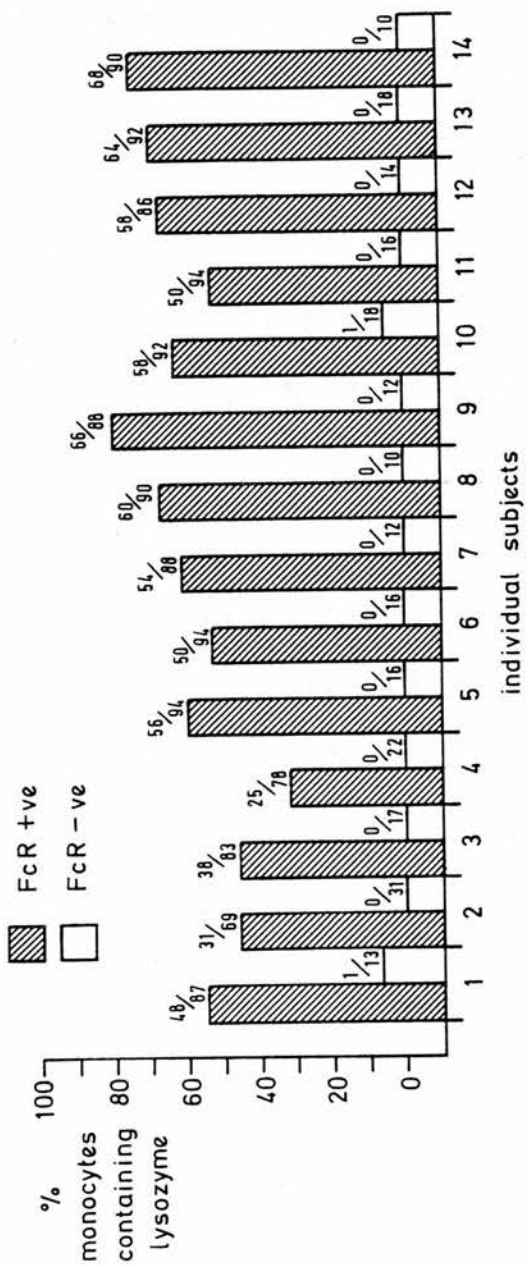


Figure 58 The percentage of Fc receptor-positive and Fc receptor-negative monocytes displaying A1AT in 14 individual blood mononuclear cell preparations.

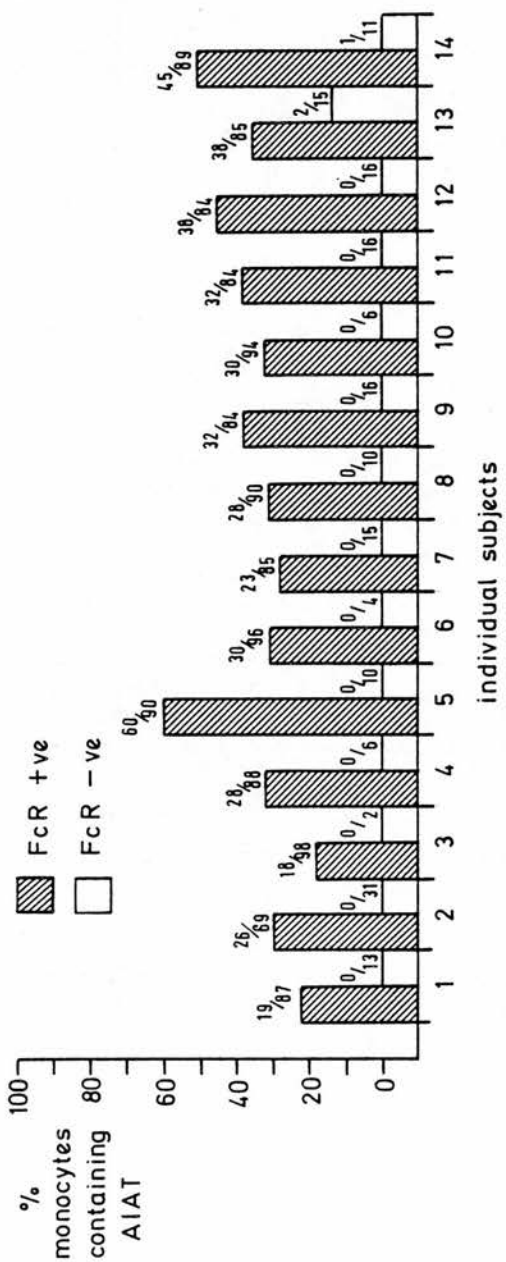


Figure 59 The percentage of C3 receptor-positive and C3 receptor-negative monocytes displaying lysozyme in 11 individual blood mononuclear cell preparations.

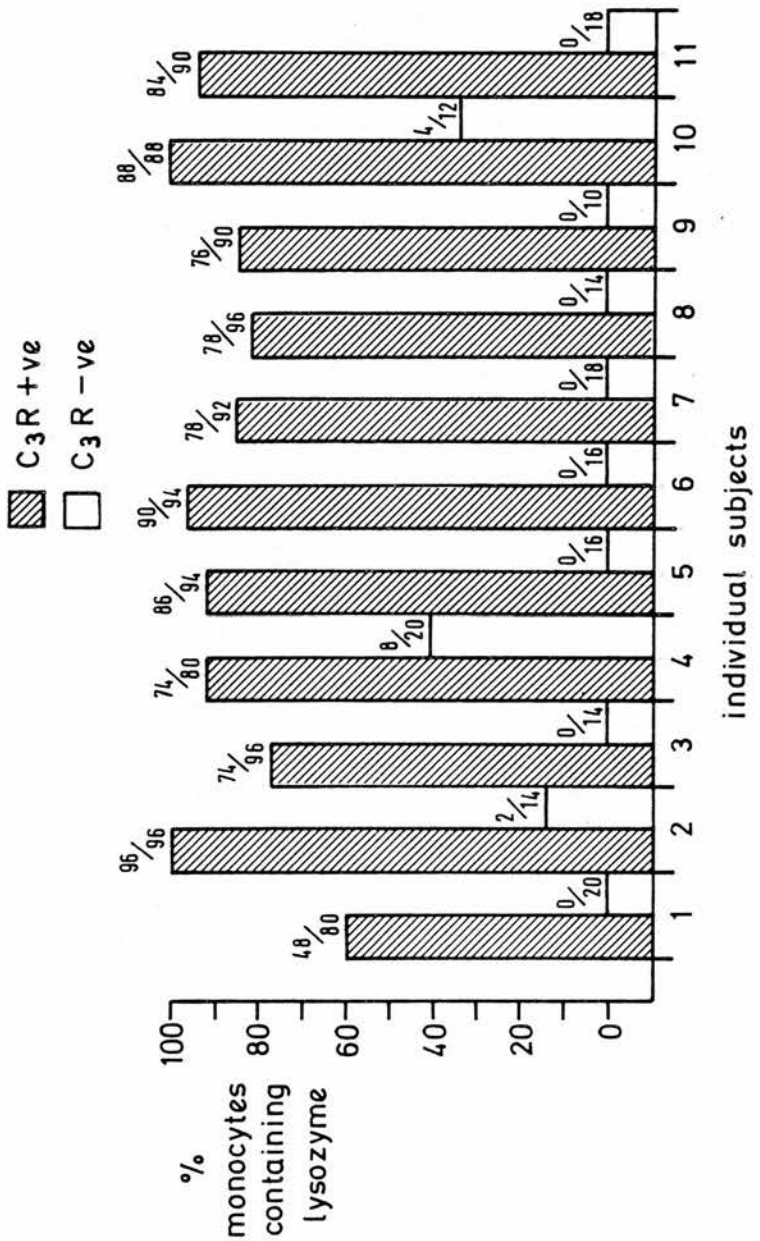


Figure 60 The percentage of C3 receptor-positive and C3 receptor-negative monocytes displaying A1AT in 11 individual blood mononuclear cell preparations.

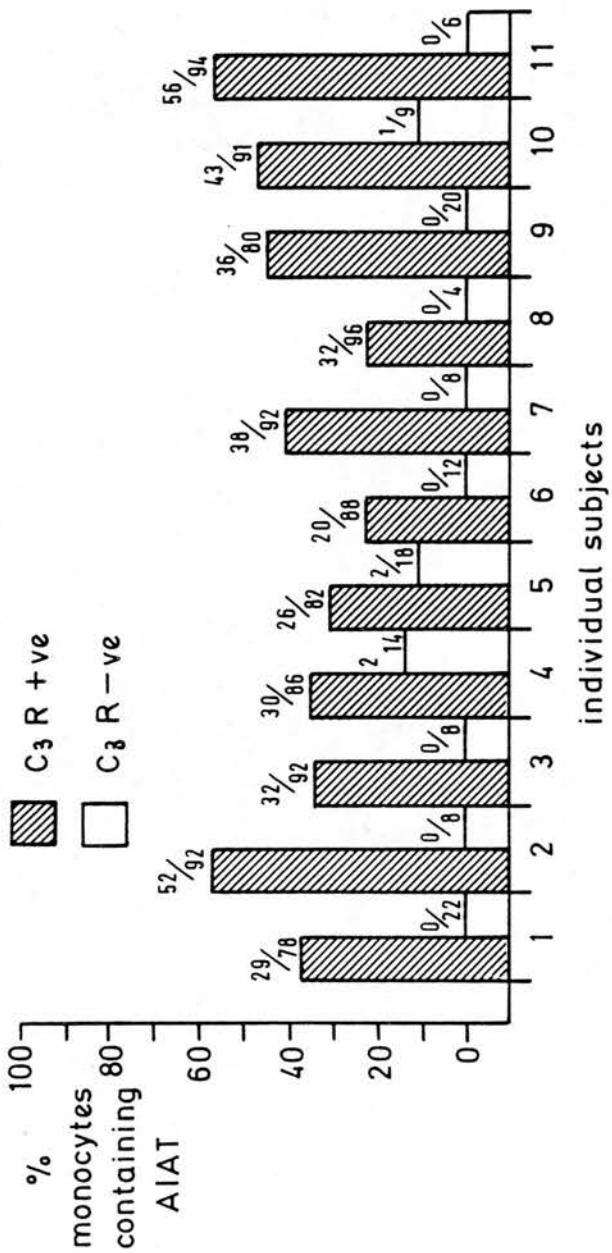


Figure 61 Comparisons between mean EA rosette size in monocytes positive and negative for lysozyme or A1AT.

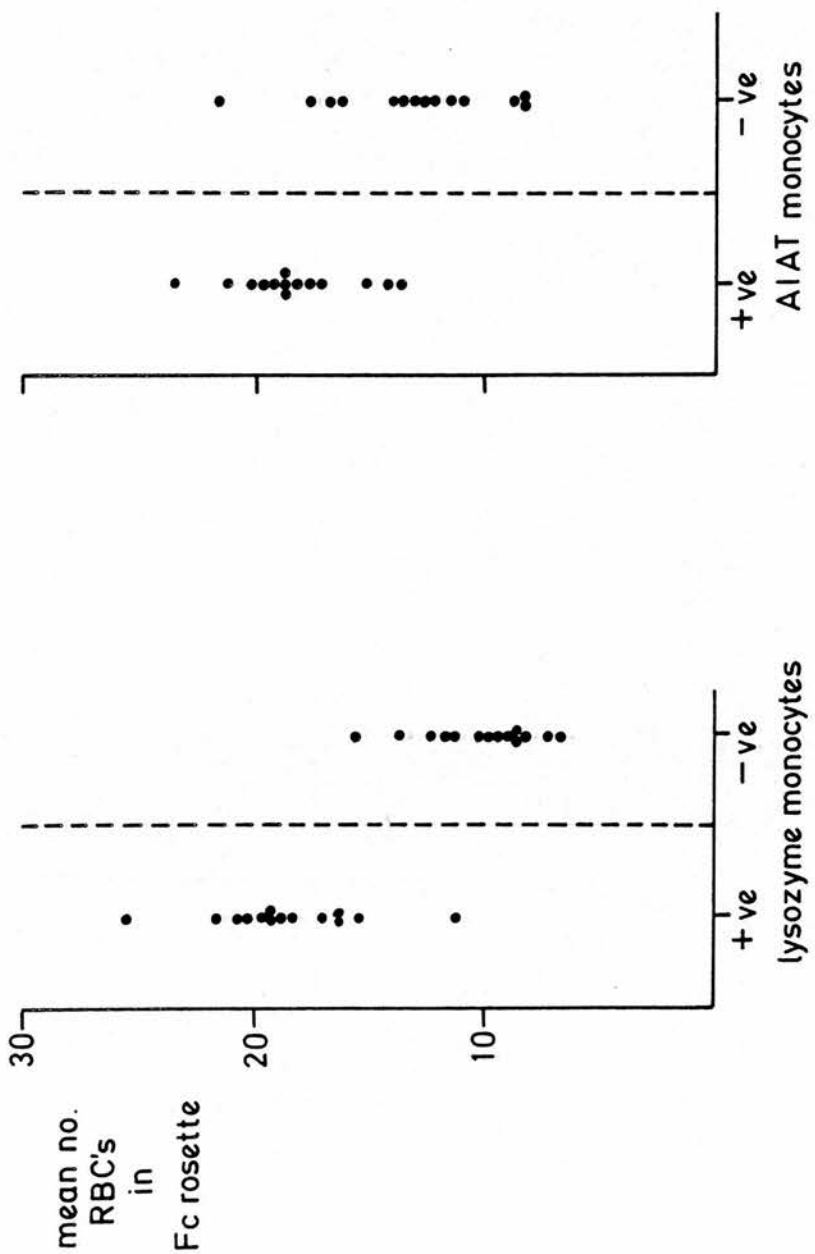
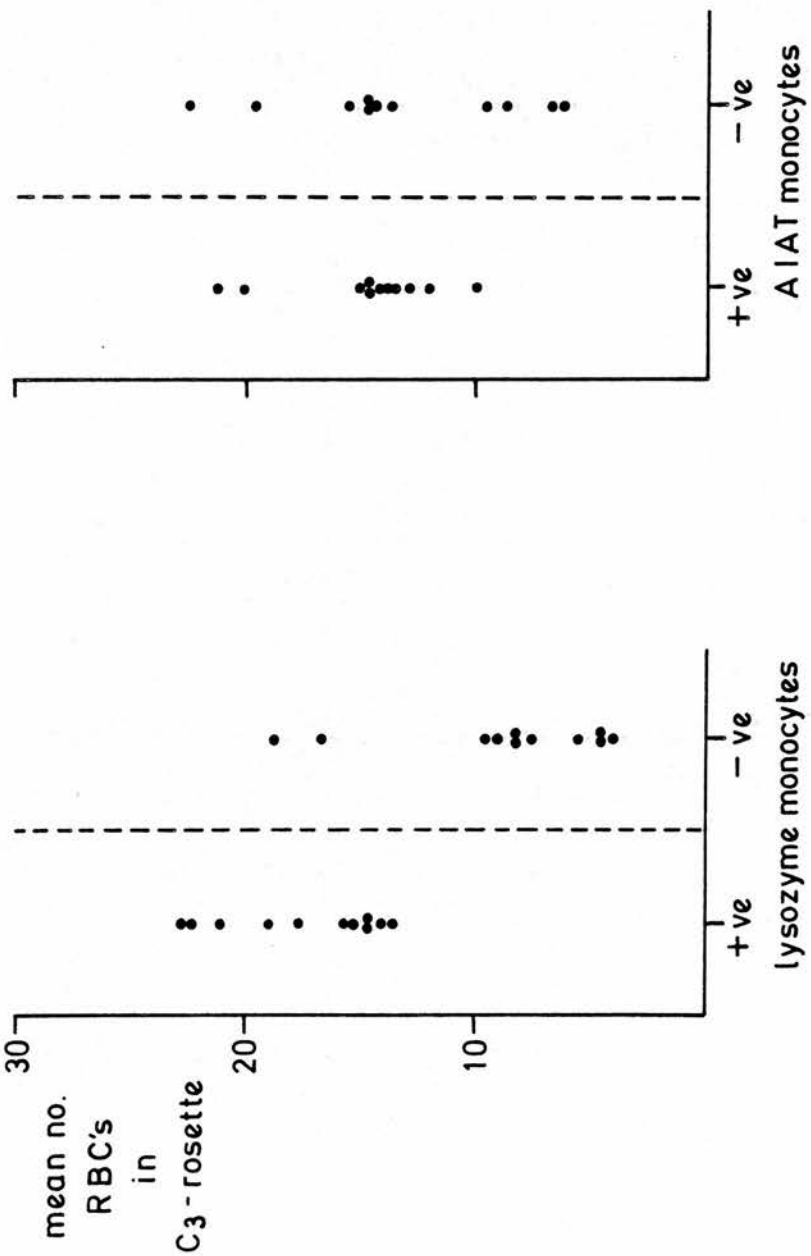


Figure 62 Comparisons between mean EAC rosette size in monocytes positive and negative for lysozyme or A1AT.



correlated with the presence of lysozyme. In essence, if lysozyme is present, then Fc and C3 receptors will almost certainly be detectable. The reverse is not true for the Fc receptor, however, as a substantial number of Fc-positive monocytes may be lysozyme negative, but in the case of the C3 receptor, most cells bearing this marker do seem to contain lysozyme, and the reason for this anomaly is explored in the next section. The degree of Fc and C3 receptor expression also seems to relate to the presence of lysozyme, and mean rosette size suggests that both receptors display heightened activity when lysozyme is present.

With A1AT a similar picture is seen, and monocytes containing this substance almost invariably display Fc and C3 receptors. However, the C3 receptor is not constantly accompanied by the presence of A1AT as was the case with lysozyme, and there does not seem to be an obvious relationship between the degree of C3 receptor activity and the presence of A1AT as measured by mean rosette size.

In conclusion, the presence of lysozyme and A1AT in monocytes tends to parallel the expression of Fc and C3 receptors, and would therefore seem to indicate heightened activation, or at least reactivity. As both of these cytoplasmic components appear to be less ubiquitous than either Fc or C3 receptors, they may prove to be more useful as indicators of the functional capacity of macrophages.

10 ii. The effect of adherence to and phagocytosis of IgG and C3 coated erythrocytes by normal human monocytes on lysozyme and A1AT content

Introduction

In the previous study, it was noted that the percentage of C3 receptor bearing monocytes containing lysozyme was much higher than for Fc receptor bearing monocytes (see Figures 57 and 59), suggesting that the formation of C3 rosettes might be exerting an influence over lysozyme content. It was therefore decided to carry out an investigation into the effect of the interaction between C3 and its receptor on the lysozyme content of monocytes, and to combine this with a wider study of the effects on lysozyme and A1AT content of adherence and phagocytosis mediated by C3 and the Fc portion of IgG.

Method

a) Patients

Venous blood samples were obtained from 14 patients who were about to undergo surgery for non-neoplastic, non-inflammatory conditions.

b) Preparation of cells

Blood mononuclear cells were isolated using a Ficoll-Hypaque gradient as previously described (q.v. pages 60 - 62).

c) Induction of opsonic adherence

Adherence mediated by the C3 or the Fc portion of IgG was attained using EAC or EA rosettes (ox erythrocytes coated by specific IgM plus C3 or specific IgG as previously described - q.v. pages 66 - 68). Phagocytosis of these attached erythrocytes was induced by incubating the rosetted blood mononuclear cells at 37°C for 2 hours, as previously described (q.v. page 140).

d) Identification of lysozyme and A1AT

Lysozyme and A1AT were detected by the immuno-peroxidase assay as previously described (q.v. pages 78 - 83), and this was carried out on cytocentrifuge preparations of

i) blood mononuclear cells

ii) blood mononuclear cells which had been incubated with indicator erythrocytes for 30 minutes at 4°C to form EA or EAC rosettes

iii) rosetted blood mononuclear cells (as above) which had been incubated at 37°C for 2 hours to induce phagocytosis of the indicator erythrocytes.

In each of these preparations, the percentage of the total monocyte population positive for lysozyme or A1AT was estimated by counting 200 cells. Cells were assessed whether or not rosetting or phagocytosis had taken place. As controls, the plain preparations of blood monocytes were also immunostained for lysozyme and A1AT after incubation at 37°C for 2 hours, and some rosetting reactions were carried out in the presence of cycloheximide (0.4 μg/ml.) to block protein synthesis.

Results

The results are illustrated in Figures 63 - 69.

a) The effect of interaction between IgG and the Fc receptor on lysozyme content

No significant alteration in the percentage of monocytes containing lysozyme was noted after formation of EA rosettes or after incubation of these rosettes at 37°C for 2 hours (Figure 63, $P > 0.1$ by Wilcoxon's signed rank test for paired data).

b) The effect of interaction between C3 and the C3 receptor on lysozyme content

After the formation of EAC rosettes, the percentage of monocytes containing lysozyme rose significantly (Figure 64, $P < 0.01$ by Wilcoxon's signed rank test for paired data), but further incubation at 37°C for 2 hours had no effect ($P > 0.1$). When EAC rosettes were formed in the presence of cycloheximide, this rise in the percentage of lysozyme-positive cells did not occur, indicating that it was abolished by blocking protein synthesis (Figure 65, $P > 0.1$). Furthermore, the formation of EAC rosettes, either in terms of percentage of positive cells or mean rosette size, was not altered by cycloheximide (Figure 66, $P > 0.1$).

c) The effect of interaction between IgG and the Fc receptor and between C3 and its receptor on A1AT content

No significant alteration in the percentage of

Figure 63 Percentage of total blood monocyte population containing lysozyme before and after formation of EA rosettes, and after phagocytosis of indicator red cells induced by 2 hours incubation at 37°C.

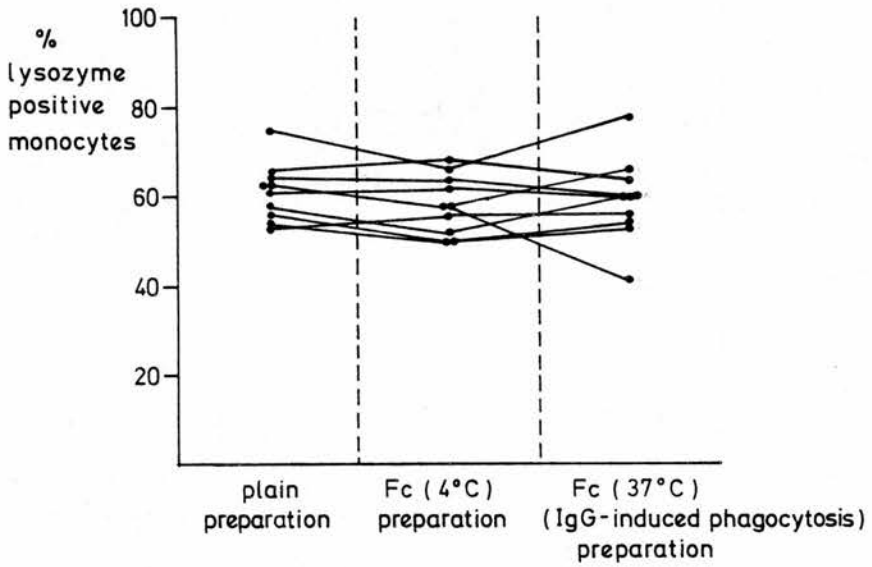


Figure 64 Percentage of total blood monocyte population containing lysozyme before and after formation of EAC rosettes, and after phagocytosis of indicator red cells induced by 2 hours incubation at 37°C.

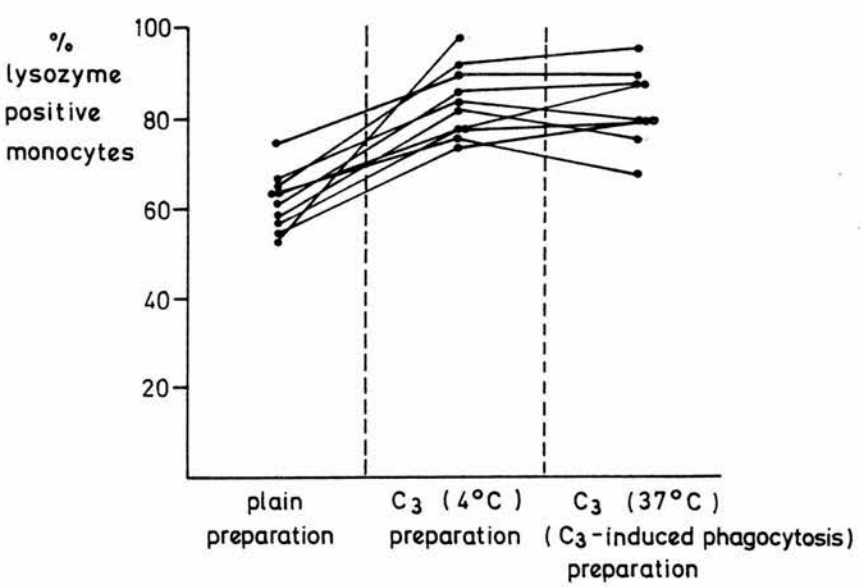


Figure 65 The effect of C3 on lysozyme content of monocytes in the presence of cycloheximide.

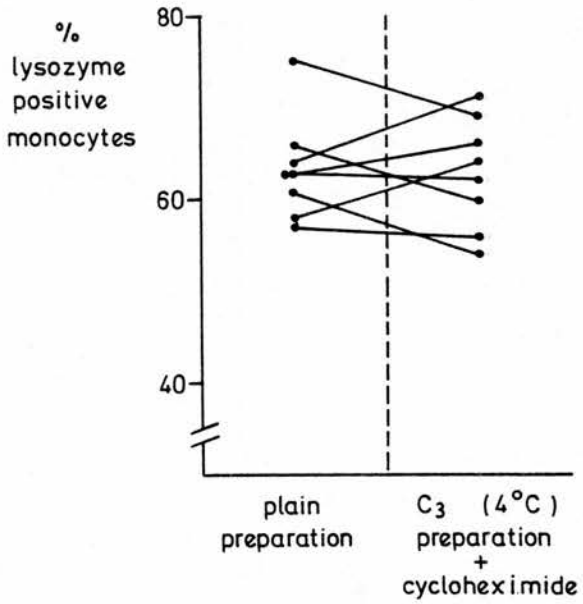
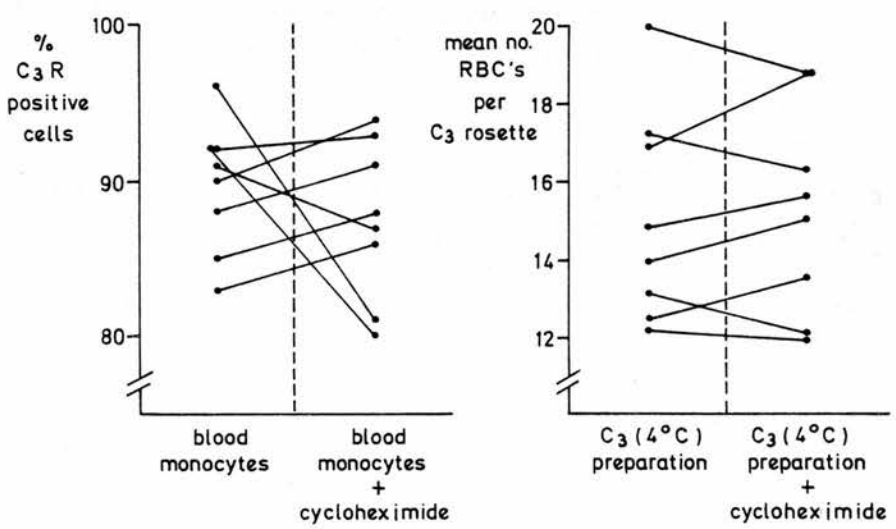


Figure 66 The effect of cycloheximide on EAC rosette formation.



monocytes containing A1AT was noted after the formation of EA or EAC rosettes (Figures 67 and 68, $P > 0.1$ by Wilcoxon's signed rank test for paired data). However, after incubation of these rosetted cells at 37°C for two hours, there was a marked decrease in the percentage of monocytes containing A1AT (Figure 67, $P < 0.01$; Figure 68, $P < 0.02$), and this drop could not be induced by similar incubation of the plain preparation of monocytes (Figure 69, $P > 0.1$).

Discussion

From these experiments, two discoveries have emerged. Firstly, interaction of C3 with its receptor on blood monocytes induced synthesis of lysozyme in the monocytes, even at 4°C, and, secondly, phagocytosis of erythrocytes, mediated by IgG or C3, depletes blood monocytes of A1AT. These findings will now be discussed in turn.

The rise in the percentage of blood monocytes containing lysozyme on reaction of these monocytes with C3-coated red cells does seem to be due to increased synthesis of the enzyme, as cycloheximide blocks the increase without inhibiting C3 receptor-detecting rosette formation. This would suggest that one function of the C3 receptor is to serve as a trigger for lysozyme synthesis, and this is not perhaps surprising, as purified C3b has been shown to increase respiration in human monocytes[193], and to stimulate the release of lysosomal enzymes from macrophages[194,195] (q.v. page 31). From the present experiments it is evident that the Fc receptor

Figure 67 Percentage of total blood monocyte population containing A1AT before and after formation of EA rosettes, and after phagocytosis of indicator red cells induced by 2 hours incubation at 37°C.

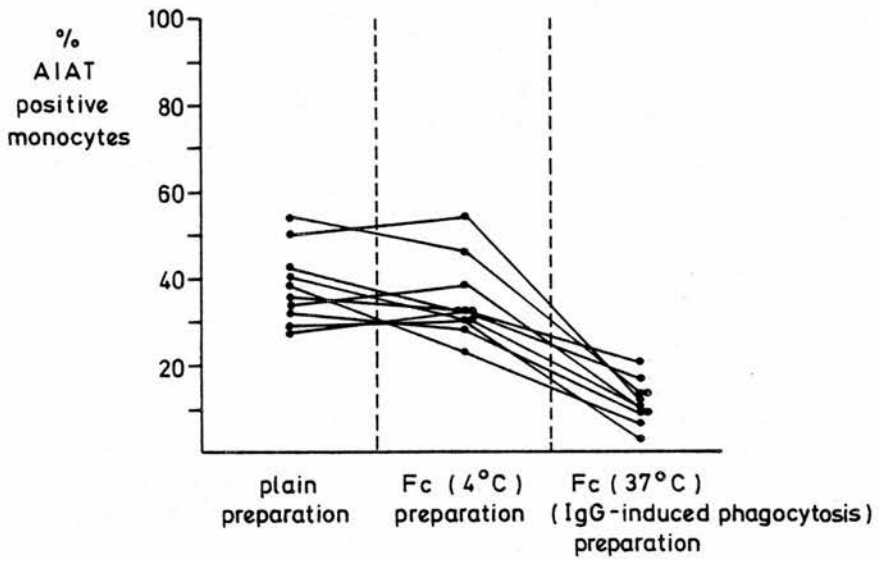


Figure 68 Percentage of total blood monocyte population containing A1AT before and after formation of EAC rosettes, and after phagocytosis of indicator red cells induced by 2 hours incubation at 37°C.

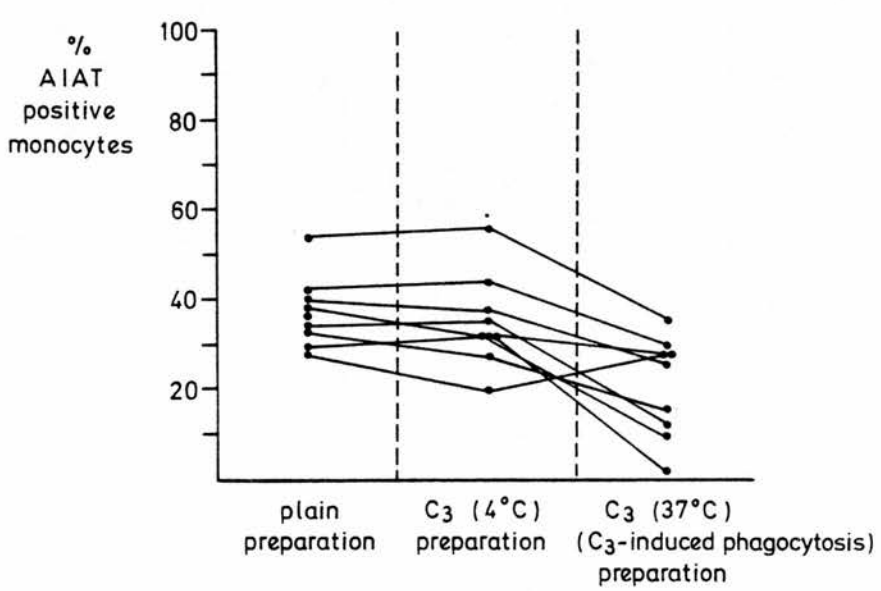
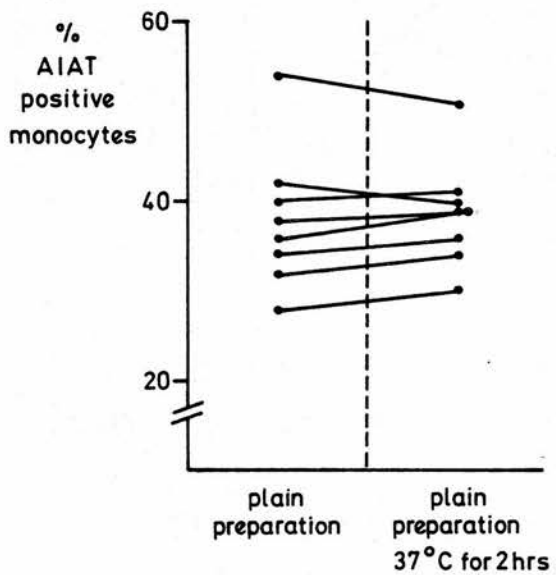


Figure 69 The effect of incubating a plain preparation of monocytes for 2 hours at 37°C on A1AT content.



has no such role to play, however, and this observation provides one possible reason for the existence of a C3 receptor as well.

C3 does not, on the other hand, appear to influence A1AT in monocytes. However, when monocytes are induced to ingest either IgG or C3 coated erythrocytes, A1AT levels appear to be reduced, suggesting that the process of phagocytosis leads to release of A1AT. As it is well recognised that phagocytosis can allow the release of various proteolytic enzymes from macrophages[214,219,258,259], it is conceivable that simultaneous release of A1AT might act as a protective mechanism against "unintentional" damage caused by leaked enzymes.

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

At the end of the discussion section of the introduction (page 53), seven questions were posed, and this discussion of the original work will be structured around these questions. Each question will be re-iterated, and the extent to which it has been answered by the experimental studies will be examined.

i) Can tumour cells display the surface markers used to identify macrophages?

This question is an important one to answer if any credence is to be placed on studies of tumour-infiltrating macrophages identified by surface markers. In section 3 (pages 92 - 100) it has been demonstrated that, in breast tumour cell suspensions, cells which express epithelial membrane antigen (EMA) do not have Fc receptors, My6C3 antigen or Ia antigen as defined by the markers which have been employed in this study. EMA does seem to be fairly specific for cells of epithelial origin[297] and is certainly expressed by breast cancer cells[296], so that these experiments provide reassuring evidence that the macrophage markers used throughout the work described in this thesis do not react with tumour cells.

ii) Is the lysozyme content of macrophages altered by the presence of tumour?

In section 4 (pages 101 - 117) it has been shown

that monocytes from breast cancer patients contain lysozyme in greater numbers than do monocytes from comparable control patients, but that tumour-infiltrating macrophages have very low levels of the enzyme. Furthermore, macrophages from lymph nodes draining breast cancers contained lysozyme in numbers which were intermediate between monocytes and tumour-infiltrating macrophages, but which were not different from control lymph nodes.

These findings have some rather interesting parallels in slightly different areas of tumour immunology. Firstly, the work of Rhodes has shown that Fc receptor expression by monocytes from cancer patients is raised when compared to normal monocytes[111,112,138], but that macrophages from the vicinity of tumours show depressed expression of this receptor[111]. This pattern closely follows the findings with lysozyme content, and, indeed, the studies on normal monocytes included in this thesis show that Fc receptor expression and lysozyme content are related (section 10, pages 161 - 170). Thus, two indices of macrophage activation are elevated in the blood but depressed in the tumour.

It has also been shown by at least three independent groups that the function of tumour-infiltrating lymphocytes is depressed whereas lymphocytes from other sites (such as blood) in the tumour-bearing host are relatively normal. This has been demonstrated for cytotoxic T-cells[298], natural killer cells[299,300], and antibody-dependent cellular cytotoxicity [300]. These findings may be of great relevance to the proposal that tumour-infiltrating macrophages are

"de-activated," as macrophages are known to have profound effects on lymphocyte function (see literature review, pages 20 - 25).

It would seem, therefore, that tumours have a certain stimulatory effect on macrophages in the peripheral blood, but that the cells which actually penetrate the tumour are markedly depressed - a pattern which is shared by some aspects of lymphocyte behaviour. It is, however, impossible to say whether this is due to in-situ depression or selective migration. It is also interesting to note that the tumour-draining lymph node macrophages were not activated in terms of lysozyme content when compared to control nodal macrophages, although they were not depressed either, and the wide range of values in the control nodes makes the results difficult to interpret. In any case, it is clear that the immediate tumour environment has an influence over host cells which is not found to the same extent in other compartments of the body.

iii) Is the A1AT content of macrophages altered by the presence of tumour?

The experiments carried out to try to answer this question are described in section 5 (pages 118 - 128), and were carried out in exactly the same manner as the lysozyme studies. The findings were also similar in that tumour-infiltrating macrophages contained A1AT less frequently than did monocytes, and nodal macrophages were also depleted compared with monocytes. However, there was no difference between control and tumour monocytes or between nodal macrophages

and tumour-infiltrating macrophages as there had been in the case of lysozyme.

It is probable that A1AT is a regulator of proteolytic enzyme activity in macrophages (see literature review, pages 42 - 46), and it is therefore likely that levels of this substance would parallel lysozyme activity. This might explain the similarities between variations in lysozyme and A1AT distribution according to the site of the macrophages, and suggests that A1AT might also be an indicator of activation. Again, study 10 i (pages 161 - 170) demonstrates a relationship between Fc receptor expression and A1AT content, supporting this latter conclusion. Why A1AT should not show the same pattern as lysozyme in control and tumour monocytes is obscure - perhaps it is just not such a sensitive activation index.

The extensive extracellular distribution of A1AT demonstrated on the tissue sections of the breast tumour (Figure 39, page 40) was an interesting and completely unexpected finding; quite different from the picture seen with lysozyme (Figure 34, page 113). This is an avenue which will be worth pursuing, with a view to investigating the histological distribution of A1AT in tumours from sites other than breast, and to finding out its role (if any) in the behaviour of the neoplasm.

iv) Do tumour-associated macrophages display altered expression of Fc receptor, C3 receptors or Ia antigens?

From the studies outlined in section 6 (pages 129 - 138), the evidence suggests that Fc receptor expression is

enhanced in the monocytes of breast cancer patients, which is in accord with Rhodes[111,112,138], but that tumour-infiltrating macrophages also have increased Fc receptor activity when compared to monocytes - a finding which does not appear to tally with Rhodes' discovery that macrophages taken from tumour-bearing lungs have depressed Fc receptor activity. However, Rhodes was studying pulmonary alveolar macrophages from different sites, and was therefore in a better position to make comparisons, as it is known that when monocytes differentiate to macrophages in vitro, increased Fc receptor expression is seen[168]. Unfortunately, it is not possible to isolate macrophages from normal breast tissue, and the comparisons between blood monocytes and tumour-infiltrating macrophages are not ideal, especially when it is considered that collagenase treatment can also cause a slight increase in Fc receptor availability. Nevertheless, the tumour macrophages did have greater Fc receptor expression than corresponding nodal macrophages, and at least one study has shown that tumour-infiltrating macrophages in mice can have increased Fc receptor expression compared to peritoneal macrophages[84]. The question of Fc receptor activity in macrophages from tumours is obviously far from resolved, but the present studies do suggest that the Fc receptor is present and functional in tumour-infiltrating macrophages, and its opsonic activity may even be enhanced. If this is true, then the lysozyme observations (section 4, pages 101 - 117) indicate that a divergence of activation parameters is taking place within a tumour, as, in normal monocytes, Fc receptor expression and lyaozyme content are related (section 10, pages 161 - 170).

Comparable findings were obtained with the C3 receptor studies. Despite collagenase treatment, tumour-infiltrating macrophages certainly have C3 receptors, and to a greater extent than nodal macrophages from the same patients. In this case, however, blood monocytes from cancer patients were not found to be substantially different from control monocytes, which is rather surprising as C3 receptors are thought of as alternative to Fc receptors[186], and might therefore be expected to vary with Fc receptors.

In contrast, the Ia antigen did not vary at all, irrespective of the source of the macrophages studied, and if tumours do have an effect on Ia antigen expression it has not been detected by the present studies.

v) Do tumour-associated macrophages have altered phagocytic activity?

In study 7 (pages 139 - 144), it was found that Fc receptor-positive macrophages from breast tumours were capable of phagocytosing IgG-coated erythrocytes, but to a lesser extent than Fc receptor-positive monocytes from the same patients. This suggests that the phagocytic mechanism may be impeded in tumour-infiltrating macrophages, as the Fc receptor activity of these cells was actually greater than that of the monocytes (see section 6, pages 129 - 138). Whether this indicates impaired anti-tumour activity per se is doubtful, as macrophage mediated tumour cell killing does not seem to involve phagocytosis[8,53,57,60], but the finding of depressed phagocytosis

in tumour-infiltrating macrophages does provide further evidence of deficient functional ability in these cells.

vi) What is the nature of sinus histiocytosis in regional lymph nodes draining tumour?

Sinus histiocytosis is a morphological feature found in lymph nodes draining tumours, and its prognostic significance is explored in Part I of this thesis (see volume 1). To summarise briefly, its presence is fairly well correlated with a good prognosis in breast cancer, but it is also closely correlated with paracortical (T-cell) hyperplasia in the regional nodes, which is a favourable factor in its own right. In addition, sinus histiocytosis can be induced by breast biopsy, indicating that it may be relatively non-specific. The relationship between sinus histiocytosis and paracortical hyperplasia, however, suggests that the former change may represent macrophages interacting with T-lymphocytes (e.g. presenting antigen), but there is no formal evidence that sinus histiocytes are, in fact, macrophages.

In study 8 (pages 145 - 151), it has been demonstrated that most sinus histiocytes bear macrophage-associated surface markers, and at least a proportion are capable of phagocytosing IgG-coated red blood cells and contain lysozyme or A1AT. It is therefore reasonable to assume that most cells which make up sinus histiocytosis are macrophages, although their function remains obscure.

vii) Does the macrophage content of a naturally occurring human tumour correlate with prognosis?

As discussed in the literature review (pages 11 - 12), the ability of different types of experimental animal tumours to metastasise was once thought to be inversely related to macrophage content, but this view has recently been strongly challenged. However, investigations into the prognostic significance of the macrophage content of a single, naturally occurring tumour type are few and far between.

In study 9 (pages 152 - 160), it has been shown that macrophage content in breast cancer does not relate to any one of four well recognised prognostic factors, but that when these factors are combined, macrophage content is higher in the poor prognostic group. This would suggest that a high macrophage content is probably an unfavourable prognostic factor, although careful and prolonged follow-up studies are required to confirm this postulate. In any case, the finding that a high macrophage content is not associated with tumours with a good prognosis accomodates the results of other studies described in this thesis, which suggest that tumour-infiltrating macrophages are at a low level of activation.

Finally, in section 10 (pages 161 - 179), some experiments investigating the function of normal monocytes are described, and from these, two conclusions emerge. Firstly, the C3 receptor acts as a trigger for lysozyme synthesis within the monocyte. However, this may be a small component of a much wider heightening of metabolic activity occasioned by C3-

monocyte interaction, as C3 has been shown to increase respiration within these cells[193]. Secondly, phagocytosis, at least of IgG and C3-coated red cells, causes depletion of A1AT from monocytes. It is not possible to say categorically that this phenomenon is due to A1AT release, but it does constitute a possible explanation. If A1AT is released by phagocytosis, and this can only be proved using an extremely sensitive assay for the substance in the supernatant of phagocytosing cells, the process might prove to be a critical homeostatic mechanism, as proteolytic enzymes are known to "leak out" of macrophages during phagocytosis[214,215,258,259]. Such a protective mechanism might have important implications for pulmonary emphysema, which is thought to be caused by elastase release from macrophages, and which is seen in smokers, and, of particular interest in this context, in patients with A1AT deficiency.

These preliminary observations on normal blood monocyte physiology offer further insight into the complex in vivo role of this cell. It is therefore hoped that their inclusion in this thesis will prove useful to future workers in this field.

APPENDIX

Contents of appendix

1. Tumour patient details

Number	Age (yrs)	Node status	OeR status	Tumour size (cms)	Tumour grade
1	47	-	+	1.5	3
2	66	+	+	1.5	2
3	66	+	+	8.0	3
4	48	+	+	7.5	3
5	62	+	+	6.5	3
6	45	+	-	2.0	2
7	67	?	+	1.0	2
8	67	-	+	2.5	3
9	75	?	-	6.0	3
10	50	?	+	5.0	3
11	59	+	+	6.5	1
12	65	+	+	4.0	3
13	64	-	+	3.5	3
14	57	-	+	2.0	1
15	47	+	+	5.0	2
16	64	+	-	3.0	3
17	75	-	-	5.0	3
18	59	+	+	2.0	2
19	46	-	?	7.0	?
20	68	-	+	2.5	1
21	51	+	+	5.0	3
22	68	+	+	8.0	3
23	63	+	+	1.3	2
24	61	-	+	3.0	3
25	64	+	+	4.0	3
26	58	-	+	2.5	2
27	49	-	-	3.0	3
28	64	+	+	3.5	2
29	42	-	+	1.3	1
30	61	+	+	6.0	3
31	58	-	-	4.0	3
32	53	+	+	4.0	3

Number	Age (yrs)	Node status	OeR status	Tumour size (cms)	Tumour grade
33	47	+	+	2.0	2
34	59	+	-	2.5	3
35	61	+	+	0.5	1
36	77	?	+	5.0	3
37	77	-	+	5.5	2
38	48	-	+	1.9	3
39	51	+	+	3.5	3
40	70	-	+	1.5	2

2. Control patient details (blood samples)

Number	Age (yrs)	Disease process
1	44	Haemorrhoids
2	57	Ovarian cyst (benign)
3	63	Ovarian cyst (benign)
4	51	Benign mammary dysplasia
5	59	Benign mammary dysplasia
6	47	Varicose veins
7	65	Varicose veins
8	68	Gall stones
9	71	Femoral hernia
10	53	Fibroadenoma
11	61	Incisional hernia
12	60	Benign mammary dysplasia
13	67	Gall stones
14	58	Benign mammary dysplasia

Control patient details (lymph node samples)

Number	Age (yrs)	Disease process	Site of node
1	47	Varicose veins	Groin
2	65	Varicose veins	Groin
3	67	Femoral hernia	Groin
4	59	Varicose veins	Groin
5	57	Gall stones	Cystic duct
6	63	Gall stones	Cystic duct
7	53	Varicose veins	Groin
8	75	Sigmoid volvulus	Sigmoid mesentery
9	58	Varicose veins	Groin
10	60	Femoral hernia	Groin

3. Macrophage lysozyme content - expressed as percentage of rosetting cells containing detectable lysozyme.

My - cells positive for My6C3 rosetting reaction (excluding lymphocytes)

Fc - cells positive for EA rosetting reaction (excluding lymphocytes)

Ia - cells positive for BT2/9 rosetting reaction (excluding lymphocytes)

a) Tumour patients

Number	Blood			Node			Tumour		
	My	Fc	Ia	My	Fc	Ia	My	Fc	Ia
1	58	59	-	7	12	-	18	15	-
2	76	83	-	-	-	-	0	-	-
3	68	66	65	7	19	10	2	14	10
4	73	74	-	-	-	-	4	5	-
5	70	70	-	13	4	-	3	3	-
6	76	74	-	4	12	-	0	0	-
7	75	77	-	11	12	12	5	4	4
8	70	75	74	8	18	10	4	8	4
9	75	76	73	12	17	15	8	8	-
10	86	80	75	-	-	-	0	2	8
11	89	83	83	-	-	-	24	24	0
12	83	72	78	16	20	25	8	8	12
13	92	92	89	23	29	21	6	5	4
14	91	93	80	24	28	24	4	6	6
15	87	87	76	8	8	8	10	11	8
16	94	94	90	15	31	10	10	14	12

b) Control patients

Number	Blood		
	My	Fc	Ia
1	59	55	-
2	48	46	-
3	19	46	-
4	28	32	-
5	46	60	51
6	52	53	57
7	56	61	60
8	63	67	60
9	71	80	66
10	64	63	71
11	53	53	56
12	66	67	68
13	64	69	74
14	63	75	68

Number	Node		
	My	Fc	Ia
1	4	10	3
2	0	9	4
3	4	6	4
4	0	0	0
5	15	8	22
6	13	20	15
7	32	41	38
8	5	6	9
9	7	15	8
10	10	19	12

4. Macrophage A1AT content - expressed as a percentage of rosetting cells containing detectable A1AT.

a) Tumour patients

Number	Blood			Node			Tumour		
	My	Fc	Ia	My	Fc	Ia	My	Fc	Ia
1	36	38	-	0	13	-	38	12	-
2	59	35	-	-	-	-	4	-	-
3	53	50	35	6	11	7	20	24	27
4	63	38	-	-	-	-	36	24	-
5	56	50	-	4	10	-	10	22	-
6	29	37	-	2	13	-	4	7	-
7	33	29	-	13	10	5	12	8	14
8	29	20	26	13	13	10	8	8	20
9	26	24	27	16	22	18	32	20	-
10	20	17	22	-	-	-	24	20	17
11	44	36	36	-	-	-	20	16	12
12	37	36	28	25	22	22	12	18	16
13	34	31	30	17	14	14	13	10	8
14	46	37	36	20	28	16	8	10	11
15	28	24	21	16	19	19	14	18	19
16	49	39	49	11	13	8	12	10	12

b) Control patients

Number	Blood		
	My	Fc	Ia
1	-	23	-
2	-	30	-
3	21	18	-
4	31	32	-
5	42	60	53
6	33	31	37
7	38	28	29
8	35	31	32
9	33	38	27
10	38	32	36
11	38	38	35
12	43	43	17
13	41	35	41
14	57	50	49

Number	Node		
	My	fc	Ia
1	2	5	2
2	4	13	4
3	8	5	2
4	28	53	6
5	12	4	5
6	0	0	0
7	28	28	27
8	24	19	18
9	16	18	15
10	19	21	21

5. Macrophage surface characteristics

i. My6C3

Values expressed as percentages of monocyte/macrophage cells positive for My6C3 rosetting reaction, and as the mean number of red cells forming an individual rosette. In the case of tumours, the percentage values refer to all cells excluding lymphocytes.

a) Tumour patients

Number	Blood		Node		Tumour	
	%	Mean	%	Mean	%	Mean
1	94	14.3	97	12.1	54	14.3
2	96	16.2	-	-	39	18.9
3	98	15.4	92	18.7	66	18.7
4	98	8.1	-	-	43	18.2
5	100	10.7	94	12.6	49	14.3
6	99	8.3	92	15.3	38	15.6
7	96	15.9	95	13.1	44	14.8
8	96	16.7	95	14.2	42	17.9
9	93	18.9	97	18.9	48	18.2
10	97	15.4	-	-	46	19.6
11	95	18.9	-	--	28	20.1
12	98	18.7	97	19.1	54	18.3
13	95	12.1	93	12.2	56	14.9
14	97	13.8	97	10.1	44	18.7
15	100	16.1	93	8.6	64	18.6
16	96	14.2	95	8.9	51	17.2
17					48	
18					32	
19					45	
20					55	
21					33	
22					64	
23					32	

/over

Number	Tumour %
24	30
25	43
26	54
27	19
28	50
29	20
30	56
31	41
32	24
33	46
34	51
35	26
36	33
37	28
38	45
39	17
40	27

b) Control patients

Number	Blood		Node	
	%	Mean	%	Mean
1	97	9.7	98	18.7
2	95	18.6	97	11.0
3	98	14.3	94	14.8
4	98	15.1	96	18.9
5	96	19.2	91	19.7
6	97	15.7	95	18.6
7	99	14.3	99	12.8
8	97	13.1	97	13.9
9	97	12.8	100	13.8
10	96	14.1	98	18.2
11	97	15.6		
12	98	14.3		
13	95	14.1		
14	92	13.2		

ii. Fc

Values expressed as percentages of monocyte/macrophage cells positive for EA rosetting reaction, and as the mean number of red cells forming an individual rosette. In the case of tumours, the percentage values refer to all cells excluding lymphocytes.

a) Tumour patients

Number	Blood		Node		Tumour	
	%	Mean	%	Mean	%	Mean
1	95	14.0	60	7.9	51	15.2
2	82	12.9	-	-	-	-
3	94	18.0	74	6.2	45	10.7
4	92	15.5	-	-	41	10.9
5	92	10.5	71	9.2	30	11.2
6	95	12.7	69	12.4	25	13.8
7	96	10.4	82	7.9	42	21.2
8	96	15.3	81	7.5	41	23.1
9	91	20.8	90	15.8	45	26.6
10	90	18.6	-	-	46	22.1
11	91	19.6	-	-	29	22.9
12	99	21.9	83	17.2	49	22.3
13	96	16.3	82	12.2	44	20.5
14	93	14.6	87	14.5	37	17.5
15	98	16.7	96	18.3	60	20.2
16	95	17.6	91	16.5	49	20.4
17					40	
18					30	
19					41	
20					51	
21					34	
22					58	
23					26	

/over

Number	Tumour %
24	22
25	38
26	41
27	12
28	46
29	15
30	59
31	40
32	18
33	39
34	51
35	21
36	40
37	24
38	38
39	18
40	25

b) Control patients

Number	Blood		Node	
	%	Mean	%	Mean
1	87	13.9	76	9.8
2	69	16.0	52	9.5
3	83	10.7	70	8.9
4	78	9.1	51	7.3
5	92	9.4	78	11.4
6	95	14.7	72	10.3
7	87	13.6	75	13.6
8	90	16.6	40	9.7
9	86	17.4	76	10.6
10	93	14.9	81	9.1
11	89	18.8		
12	85	17.1		
13	88	18.0		
14	90	15.8		

iii. C3

Values expressed as percentage of monocyte/macrophage cells positive for EAC rosetting reaction, and as the mean number of red cells forming an individual rosette. In the case of tumours, the percentage values refer to all cells excluding lymphocytes.

a) Tumour patients

Number	Blood		Node		Tumour	
	%	Mean	%	Mean	%	Mean
1	54	5.9	-	-	-	-
2	44	4.3	-	-	-	-
3	94	11.0	76	10.7	24	7.5
4	80	11.7	-	-	20	5.1
5	90	8.6	60	8.2	18	9.8
6	86	15.6	54	6.3	7	10.5
7	93	10.6	53	8.3	21	8.5
8	97	15.4	60	5.4	27	10.2
9	92	18.3	59	6.1	31	16.4
10	87	17.7	-	-	22	6.5
11	95	17.0	-	-	16	8.7
12	93	15.9	54	7.7	38	10.4
13	94	16.1	65	7.6	18	8.2
14	98	16.8	76	9.4	11	11.6
15	96	15.8	49	7.1	32	9.1
16	95	14.0	51	9.7	28	13.2

b) Control patients

Number	Blood		Blood and cyclohex		Node	
	%	Mean	%	Mean	%	Mean
1	-	-	-	-	38	6.1
2	-	-	-	-	20	10.6
3	-	-	-	-	26	7.2
4	80	13.3	-	-	28	11.8
5	94	22.8	-	-	60	7.8
6	95	11.0	-	-	72	12.9
7	83	14.9	86	15.7	62	6.3
8	88	20.4	91	18.9	65	14.9
9	91	17.3	87	16.4	66	5.8
10	92	17.0	93	18.9	68	5.1
11	96	12.5	81	13.6		
12	85	12.2	88	12.0		
13	90	14.0	94	15.1		
14	92	13.2	80	12.1		

iv. Ia

Values expressed as percentage of monocyte/macrophage cells positive for BT2/9 rosetting reaction, and as the mean number of red cells forming an individual rosette. In the case of tumours, the percentage values refer to <u>all</u> cells excluding lymphocytes.

a) Tumour patients

Number	Blood		Node		Tumour	
	%	Mean	%	Mean	%	Mean
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	90	22.0	99	22.0	43	21.6
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	91	14.3	45	14.5
8	95	20.8	96	14.3	39	18.5
9	97	24.0	98	19.8	-	-
10	94	19.1	-	-	48	22.7
11	98	26.2	-	-	32	23.8
12	100	21.2	94	20.4	56	26.0
13	98	17.6	98	18.3	55	15.2
14	99	21.8	97	23.9	48	20.6
15	100	19.0	93	14.9	61	18.0
16	93	14.4	94	18.1	54	22.2
17					40	
18					38	
19					50	
20					55	
21					36	
22					60	
23					36	

/over

Number	Tumour %
24	32
25	45
26	54
27	24
28	49
29	22
30	55
31	39
32	21
33	47
34	49
35	25
36	38
37	29
38	43
39	20
40	28

b) Control patients

Number	Blood		Node	
	%	Mean	%	Mean
1	-	-	100	19.6
2	-	-	100	20.5
3	-	-	96	13.6
4	-	-	98	18.2
5	93	20.7	92	19.7
6	97	17.9	100	19.5
7	96	18.9	97	22.3
8	100	25.4	98	23.1
9	100	24.7	95	20.8
10	99	22.9	97	16.9
11	92	20.1		
12	95	19.8		
13	94	19.3		
14	100	18.5		

6. Phagocytosis of IgG-coated red cells

Values expressed as percentages of Ea-rosetting cells phagocytosing red cells after incubation for 2 hours at 37°C.

a) Tumour patients

Number	Blood	Node	Tumour
5	-	59	-
6	-	38	-
7	57	8	30
8	49	60	22
9	73	24	18
10	72	-	56
11	62	-	48
12	73	32	40
13	77	12	40
14	50	30	36
15	48	24	20
16	37	28	25

b) Control patients

Number	Blood	Node
1	-	8
2	-	10
3	-	12
4	-	32
5	47	35
6	46	21
7	45	18
8	48	17
9	44	27
10	55	35
11	74	-
12	56	-
13	56	-
14	44	-

7. Relationship between Fc and C3 receptor expression on monocytes and lysozyme and A1AT content

Values expressed as mean number of red cells forming an individual rosette.

a) Lysozyme

Number	Lysozyme positive cells		Lysozyme negative cells	
	Fc	C3	Fc	C3
1	18.4	-	8.8	-
2	18.6	-	13.8	-
3	17	-	7.3	-
4	16.3	15.2	8.9	7.8
5	11.2	22.9	6.9	4.3
6	19.2	21.1	10.1	8.1
7	16.4	14.6	9.8	4.3
8	19.7	22.3	8.1	18.9
9	19.3	17.8	8.3	16.9
10	15.5	19.0	11.8	8.1
11	25.5	14.6	12.1	9.7
12	20.1	15.7	15.7	4.0
13	21.7	14.0	11.2	9.1
14	20.6	13.8	9.3	5.5

b) A1AT

Number	A1AT positive cells		A1AT negative cells	
	Fc	C3	Fc	C3
1	18.1	-	14.1	-
2	19.2	-	11.1	-
3	21.3	-	12.7	-
4	14.1	14.1	8.2	6.9
5	15.2	14.6	8.1	8.8
6	20.2	12.1	11.6	9.5
7	13.8	10.1	12.3	6.3
8	18.3	21.3	9.7	19.7
9	17.8	15.0	16.1	22.2
10	17.2	20.1	16.7	15.3
11	23.8	13.6	21.7	14.9
12	18.9	13.0	17.8	13.9
13	19.6	14.7	12.9	14.9
14	18.7	13.9	13.6	14.7

8. Comparison between untreated blood monocytes and monocytes reacted with EA or EAC in terms of percentages containing lysozyme or A1AT.

Fc - monocytes forming EA rosettes at 4°C

C3 - monocytes forming EAC rosettes at 4°C

FcP - monocytes phagocytosing EA after 2 hours incubation at 37°C

C3P - monocytes phagocytosing EAC after 2 hours incubation at 37°C

a) Lysozyme content

Values expressed as percentages of total monocytes containing lysozyme.

Number	Untreated	Fc	C3	FcP	C3P	C3 and cyclohex
5	53	56	98	56	-	-
6	54	50	74	55	80	-
7	58	54	82	60	76	64
8	61	62	86	60	88	54
9	75	66	90	78	90	69
10	63	58	78	66	80	62
11	57	50	78	54	88	56
12	63	58	76	42	68	66
13	64	64	92	60	90	71
14	66	68	84	64	80	60

b) A1AT content

Values expressed as percentages of total monocytes containing A1AT.

Number	Untreated	Untreated Incubated at 37°C for 2 hrs.	Fc	C3	FcP	C3P
5	50	-	54	52	11	-
6	29	-	30	32	2	10
7	38	39	23	32	6	2
8	32	34	28	28	10	16
9	28	30	32	20	16	28
10	40	41	30	38	10	26
11	36	39	32	32	12	28
12	34	36	38	36	8	12
13	42	40	32	44	20	30
14	54	51	46	56	12	36

Reference numbers for figures

Figure number	Reference number (Department of Medical Illustration, University of Edinburgh)
1	-
2	21283/C
3	21283/B
4	21283/E
5	21283/D
6	R7791-10
7	R7791-8
8	21283/F
9	R7791-7
10	22298/T
11	22298/S
12	22298/Q
13	22298/P
14	22298/R
15	22298/I
16	22298/H
17	-
18	-
19	-
20	-
21	22298/AP
22	22298/AO

Figure number	Reference number
23	22298/AN
24	-
25	-
26	-
27	-
28	-
29	-
30	-
31	22298/AL
32	22298/AM
33	22298/AK
34	-
35	-
36	22298/AI
37	22298/AJ
38	22298/AH
39	-
40	-
41	22298/G
42	22298/F
43	22298/E
44	22298/D
45	22298/C
46	22298/B
47	-

Figure number	Reference number
48	-
49	22298/AG
50	-
51	22298/AF
52	22298/V
53	22298/Y
54	22298/W
55	22298/U
56	22298/X
57	22298/AE
58	22298/AD
59	22298/AC
60	22298/AB
61	22298/AA
62	22298/Z
63	22298/A
64	22298/O
65	22298/N
66	22298/M
67	22298/L
68	22298/K
69	22298/J

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