FLOW CYTOMETRIC ANALYSIS OF THE LYMPHOCYTE POPULATIONS IN PATIENTS WITH BREAST CARCINOMA

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For Hans and all my family

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ABBREVIATIONS

AET	aminoethylisothiouronium bromide
BSA	bovine serum albumin
EBV	Epstein Barr virus
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter of laser light
FL.1,2,3	fluorescent channel 1, 2 or 3
HLA	human lymphocyte antigen
IgG	immunoglobulin G
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
Kbp	kilo base pairs
LNLs	lymph node lymphocytes
MHC	major histocompatibility complex/antigen
PBLs	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PHA	phytohaemagglutinin
PI	propidium iodide
PMA	phorbol myristate acetate

RPMI	Rosewell Park Memorial Institute
SRBCs	sheep red blood cells
SSC	Side scatter of reflected laser light
Tac	T activation, synonym for the CD25 antigen on the beta chain of the interleukin 2 receptor
TBS	Tris buffered saline
TILs	tumour infiltrating lymphocytes
Trf	transferrin
TrfR	transferrin receptor

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STATEMENT OF COLLABORATION

The study design was constructed by myself with advice from Dr. A.M. Campbell.

The samples of tumour, lymph node and blood were initially collected and processed by myself. I was assisted by Mr. P. Ferry as the project progressed.

The flow cytometric studies were performed by myself.

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SUMMARY

Evidence of antigen recognition and an anti-tumour immune response has been sought in patients with breast carcinoma since a lymphocytic infiltrate was first noted in the primary tumours and found to correlate with an improved prognosis [384].

A variety of methods have been used to study immune competence but, with greater understanding of lymphocyte function, a clearer picture can be obtained by analysing the phenotypic proportions present in any lymphocyte population.

The activation status of lymphocytes can also be deduced from their expression of activation markers and receptors, while the presence of surface IgG on the membrane of B cells is indicative of a mature humoral response.

The aims of this study were:

1) To seek evidence of antigen recognition and an anti-tumour immune response in patients with breast cancer.

2) To assess the contribution of the axillary lymph nodes to any humoral or cellular immune response and the extent to which any response remains loco-regional at the time of clinical presentation.

In this study, fluorescent monoclonal antibodies were used to stain the phenotypic and activation markers and flow cytometry was used to analyse the lymphocytes harvested from the primary tumours of 31 patients, the axillary lymph nodes of 40 patients and the peripheral blood of 39 patient. These were analysed with regard to the proportions of CD4+ helper T cells, CD8+ suppressor/cytotoxic T cells and B cells.
We also looked at the proportion of each cell type carrying the activation marker HLA DR, the class II major histocompatibility (MHC) antigen, and the receptors for interleukin 2 (IL-2) and transferrin (Trf). In the case of the B lymphocytes, we stained surface membrane IgG.
These parameters were also studied on the lymph node and peripheral blood lymphocytes of 7 control subjects undergoing vascular surgery or organ donation.
The tumour cells themselves were stained for the expression of the class I and class II MHC antigens and for membrane bound IgG.
The phenotypic proportions and activation status of the

tumour infiltrating lymphocytes (TILs) were correlated with the tumour cell expression of class I and class II MHC antigens and with the common prognostic indicators of tumour stage, histological tumour grade and oestrogen receptor status.

The composition and activation status of the lymph node and peripheral blood lymphocytes were correlated with tumour stage.

PRIMARY TUMOUR

TILs were found in 85% of the primary breast tumours studied and, in 60%, the infiltrate was sufficient to allow analysis of both the phenotypic and activation markers.

T cells made up the bulk of the infiltrate with few B cells. CD8+ suppressor/cytotoxic T cells predominated overall, although CD4+ helper T cells were in the majority in 7 tumours with a strong infiltrate. The size of the CD8+ T cell population increased with histological grade ($p<0.05$) and tumour cell expression of the class I MHC complex ($p<0.01$).

A large proportion of the CD8+ T cells were found to carry HLA DR and again this proportion increased with histological grade ($p=0.003$) and the tumour cell expression of both the class I and class II MHC

complexes ($p<0.001$). These findings suggest that activated CD8+ suppressor/cytotoxic T cells are being attracted by surface antigen, on poorly differentiated tumours, when this is presented along with the class I MHC complex. This latter is necessary for antigen recognition by CD8+ T cells.

A similar pattern was seen with the CD4+ helper T cells but to a slightly lesser degree.

However, a higher proportion of the CD4+ T cells than CD8+ T cells bore the receptors for interleukin 2 ($p<0.0001$) and transferrin, suggesting there is greater turnover or expansion of this cell type.

There appears therefore to be some evidence, within the primary tumour, of the recognition of antigen and a cellular immune response to it.

AXILLARY LYMPH NODES

Lymph nodes from breast cancer patients were found to be large and engorged, with higher cell counts than nodes from control subjects.

The nodes from patients with breast cancer had a higher proportion of CD4+ helper T cells ($p=0.003$) with CD4+/CD8+ T cell ratios ranging as high as 16:1 and averaging 5:1 ($p=0.02$).

In the cancer nodes, a higher percentage of the T cells

bore HLA DR ($p<0.0001$) and, in the case of the CD8+ T cells this correlated with tumour stage ($p=0.02$). This again reinforces the association of this marker with the direct interaction of CD8+ T cells with antigenic tumour cells, in this instance, within nodal metastases.

The IL-2 receptor was present on a greater proportion of the lymph node lymphocytes (LNLs) in the breast cancer patients ($p=0.006$) and, as in the TILs, this receptor was present on more CD4+ than CD8+ T cells.

Although the transferrin receptor was found on more lymphocytes in the nodes of breast cancer patients this was not statistically significant.

Almost double the number of B cells were found to carry surface IgG in the breast cancer nodes as in the control nodes ($p=0.03$).

There is evidence of helper T cell expansion and a humoral immune response in the axillary lymph nodes of patients with breast cancer. The CD8+ T cells appear more activated in patients with nodal metastases.

PERIPHERAL BLOOD

Apart from a slightly smaller B cell population, there was little difference in the phenotypic proportions of peripheral blood from patients with breast carcinoma and normal subjects.

HLA DR was found on a much greater proportion of the T cell population, of both phenotypes, in the peripheral blood of breast cancer patients compared to controls ($p<0.0001$).

While more of the CD8+ T cells in the blood of cancer patients bore the IL-2 receptor ($p<0.03$), the increase in the expression of this marker within the CD4+ T cell population was even greater ($p=0.006$). And it was again found on more CD4+ T cells than CD8+ T cells.

The cancer patients also had a larger IgG bearing B cell population ($p=0.003$) than the controls.

When the different lymphocyte sources were compared, the axillary nodes were found to be the major site of the CD4+ helper T cells ($p<0.03$) and the B cells ($p<0.001$) while the primary tumour appeared to be the most relevant source of the CD8+ suppressor/cytotoxic T cells ($p<0.001$). Although the peripheral blood lymphocytes of cancer patients showed some activation, when compared with controls, the immune response still appeared to be largely loco-regional at the time of presentation.

The peripheral blood was found to reflect the T cell subset proportions ($p<0.001$) and the size of the IgG bearing B cell population ($p<0.001$), within the axillary nodes and analysis of peripheral blood might give some indication of the immune status of the axillary nodes.

HUMAN MONOCLONAL ANTIBODIES

In an attempt to characterize the IgG produced by the nodal B cells, in many patients with breast cancer, we tried to immortalize them using EBV transformation, cell fusion or a combination of the two.

While we did detect some immunoglobulin binding on sections of autologous tumour, we had no success in producing human monoclonal antibodies and detailed study of this humoral immune response was therefore not possible.

CONCLUSION

This study provides some evidence of antigen recognition and a loco-regional immune response, both cellular and humoral, in many patients with breast carcinoma. This is particularly evident in those patients with a malignancy of high histological grade and tumour cell expression of the class I major histocompatibility complex.

CHAPTER 1: INTRODUCTION AND AIMS

> ***In mamillis saepe vidimus tumorem forma ac figura cancro animali exquisite consimilem. Nam quemadmodum in isto pedes ex utraque parte sunt corporis, ita in hoc morbo venae distenduntur, ac figuram omnio similem cancro representant.***
>
> Galen: De Art. Curat. Ad Glaucon.
>
> Lib. 2, Cap. 10

The term "Cancer" was coined by the Roman physician Galen around the year 200 A.D. and was derived from his description of locally advanced breast carcinoma in which the radial congested skin veins around the tumour appeared like the legs of a crab. From this time until the second half of the nineteenth century cancer was considered a systemic disease caused by melancholia, an excess of black bile, for which there was no cure. After this time the theory of breast cancer development changed and it was thought that tumours began locally, possibly due to obstruction or inflammation of a duct or gland, and then spread in an orderly fashion, via lymphatics, to the regional lymph nodes which acted as filters and from there to other organs [442]. Blood borne metastases were not considered to contribute greatly to

the spread of disease. It was upon these principles that the rationale for radical mastectomy, as advocated by Halsted [175], was based (Fig. 1). This theory of cancer progression, and the surgical management it endorsed, continued until the second half of this century, since when it has been challenged, and once again breast cancer is considered to be systemic quite early in the disease process, though developing from a local tumour rather than leading to one. The surgical management has therefore also been challenged, both by those who felt the morbidity of such radical surgery was unacceptable and of little benefit and by those who believed that the destruction of the axillary lymph nodes might actually be detrimental if they were the seat of an anti-tumour immune response.

One of the major arguements against the existence of an anti-tumour immune response is that no definitive tumour antigen has ever been identified and, as oncogenic products are largely intracellular, there may not be any surface antigen to be detected by the immune system. Alternatively, the tumour growth and antigen presentation may develop so slowly as to induce tolerance in the host [351].
While tumours of viral origin are more frequent among patients who are immunosuppressed, due to aquired immune

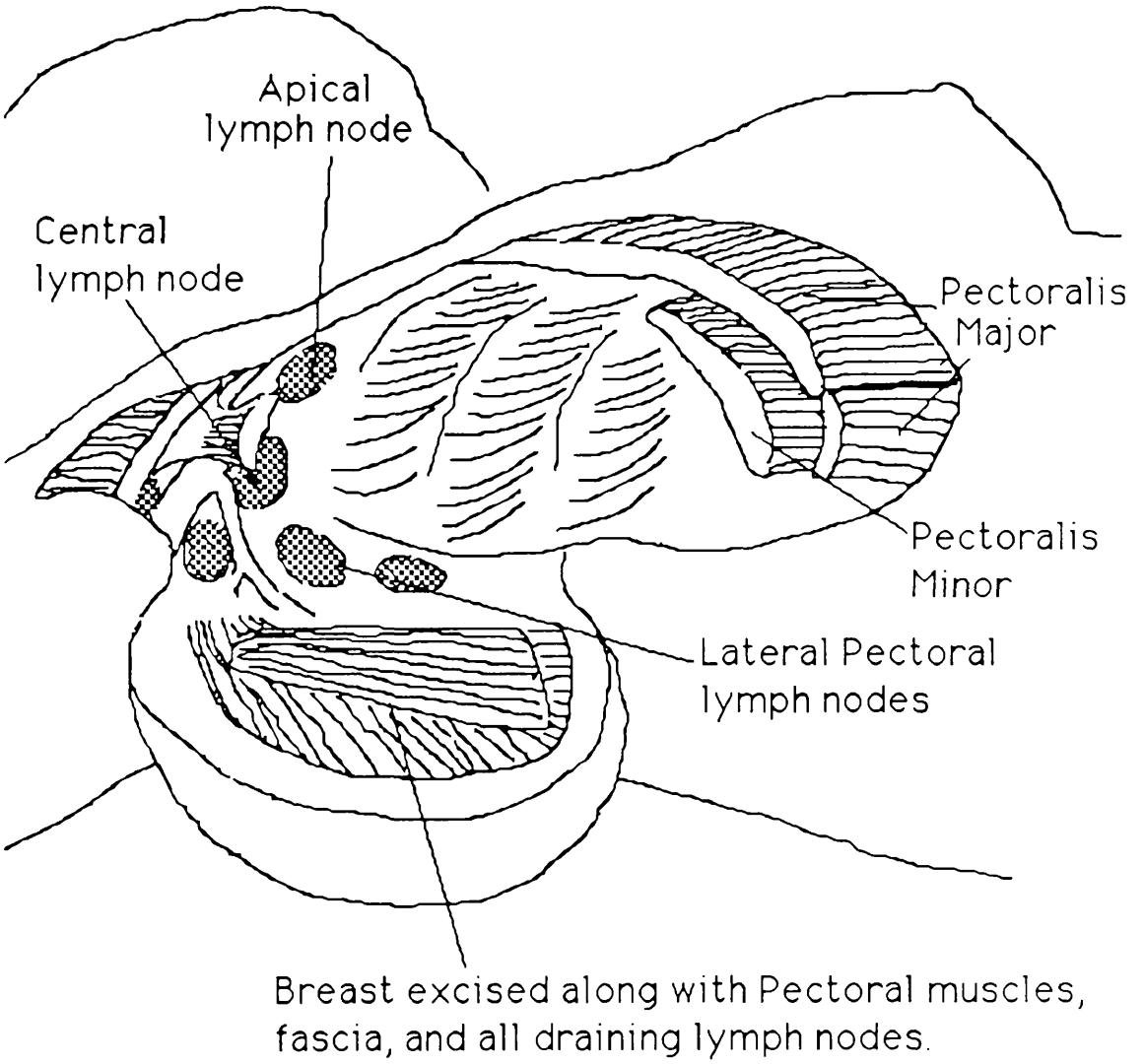


Fig. 1 The Halsted mastectomy was based on the principal that breast cancer spread in a progressive fashion up through the lymph node chains before any distant metastasis occured.

deficiency syndrome (AIDS) or long term immunosuppressive therapy after organ transplant, there is no increase in the common malignancies such as breast carcinoma [153, 327].

The possible existence of a host anti-tumour immune response is, however, supported by the long term survival of some patients known to have residual malignant disease after therapy [133] or those who develop recurrence, many years after the initial presentation, during some time of great stress, such as bereavement, which appears to depress the immune system [20, 223, 372]. The question of whether or not patients with breast cancer mount an immune response to their tumour is important for three main reasons.

If some patients mount an effective immune response against their tumours this could be expected to improve their survival and therefore evidence of that immune response might be an important prognostic indicator. Secondly, the presence of a response and its anatomical site would have to be considered when planning adjuvant therapy which affects the immune system. If that response were regionally based it would be destroyed by loco-regional therapy such as surgery or radiotherapy while a centralised immune response would be depressed by cytotoxic chemotherapy.

Lastly, if an effective immune response could be identified and the mechanisms of it understood it might be possible to enhance that response, where present, and develop methods of immunotherapy or stimulation for those patients not mounting a response.

AIMS OF STUDY

1) To seek evidence of antigen recognition and an immune response in patients with breast cancer.

This was done by studying the phenotypic subsets and surface activation markers of lymphocytes infiltrating the primary tumour of breast cancer patients and correlating these results with the degree of tumour differentiation, as represented by histological tumour grade and oestrogen receptor status with tumour cell expression of the class I and class II major histocompatibility molecules which are required for the presentation of antigenic material to the immune system and with the stage of tumour spread.
The phenotypic and activation markers were also studied on lymphocytes from the axillary lymph nodes and peripheral blood of patients with breast cancer, correlated with tumour stage and compared with lymph

node and peripheral blood lymphocytes from normal subjects.

2) To assess the contribution of the axillary lymph nodes to any humoral or cellular immune response in patients with breast cancer and the extent to which any response remains loco-regional at the time of clinical presentation.

The phenotypic subsets and surface activation markers on lymphocytes from the primary tumours, axillary lymph nodes and peripheral blood of individual patients with breast carcinoma were compared to assess the relative contribution of the axillary lymph nodes to any response and to consider the effect on this of the current management of breast cancer.
Correlation between the blood lymphocyte markers and those in the other tissues was sought as a guide to the immune status in the region of the tumour.

3) To consider the possibility of developing immunotherapeutic approaches to the management of breast cancer.

Based on the results of the above studies, potential methods of immune enhancement were considered. In

particular, the feasability of developing human monoclonal antibodies from patients with breast cancer were assessed as, if this were successful, it might allow the isolation and study of tumour antigens and provide useful tools for the diagnosis and therapy of breast cancer.

CHAPTER 2: EVIDENCE OF ANTIGEN PRESENTATION AND AN IMMUNE RESPONSE IN PATIENTS WITH BREAST CANCER

INTRODUCTION

The initial question to be answered was whether any patients with breast cancer show evidence of an immune response against their tumours.

As no tumour specific antigens have been identified or characterized, it has been suggested that there are no membrane tumour antigens to be recognized by the immune system and particular attention was therefore paid to evidence of tumour antigenicity or antigen presentation and recognition.

Lymphocytes from the axillary lymph nodes and peripheral blood of patients with breast cancer were compared with those obtained from normal subjects to demonstrate whether the breast cancer patients showed evidence of immune stimulation or suppression.

HISTORICAL REVIEW

HISTOLOGICAL STUDIES OF TUMOUR INFILTRATING LYMPHOCYTES

The first suggestion that cancer patients might mount an immune response to their tumours came in the early years of this century when Handley [180] found that the spontaneous regression of malignant skin melanomata was associated with a round cell infiltrate and Da Fano [96], from his animal studies, suggested that this infiltration of tumours by plasma cells and lymphocytes might represent an immune response as part of the host's tumour defences. This theory was supported by MacCarty & Mahle [203] who observed an improved survival among patients suffering from gastric cancer who had an inflammatory infiltrate in their tumours and demonstrated a similar survival advantage in both breast [384] and colon cancer [264]. This finding appeared to be supported by the study of medullary carcinoma which has a particularly good prognosis and often has a marked lymphocytic infiltrate [284, 93, 48, 344]. However, as Richardson [341] pointed out, medullary tumours have a better prognosis, even when the lymphocytic infiltrate is absent, due to their poor stroma formation and consequent inability to form viable metastatic deposits.

This was confirmed in the study by Ridolfi [344] when patients with medullary carcinoma were found to have the same 10 year survival rate regardless of whether or not they had a prominent lymphocytic infiltrate although this infiltrate was again associated with improved survival in patients with non-medullary carcinoma. Although the positive correlation between the presence of an inflammatory tumour infiltrate and improved survival was rather weak in these original studies, interest in it continued and in the first half of this century many studies were carried out looking at the effect of this infiltrate on survival prognosis. Eighteen studies looked at this phenomenon in breast cancer patients and thirteen of these found an improved survival rate among patients with an inflammatory infiltrate in their tumours [384, 167, 35, 27, 197, 8, 177, 92, 94, 48, 434, 38, 344], while five groups found no survival advantage for such patients [69, 287, 353, 358, 139]. Two of these latter even suggested that an inflammatory infiltrate was associated with a poor prognosis [353, 139].

Taking solid tumours as a whole, the great majority of studies found the presence of an inflammatory infiltrate to be associated with a better prognosis. Controversy continued, however, over whether this infiltrate was evidence of a host anti-tumour response [264, 384, 123] or

secondary to tumour necrosis which was in itself due to other factors [167, 207, 99]. As a prognostic factor, inflammatory infiltrate did not appear to be as strong as histological tumour grade or stage and so its relevance remained unclear [45, 46, 47, 73].
These early studies merely noted the presence or absence of lymphocytes within the tumour, using conventional haematoxylin and eosin stains, but were unable to differentiate the cell types involved.

ANIMAL STUDIES

In an attempt to confirm the antigenicity of tumours and the existence of an anti-tumour immune response, many workers turned to animal models. The commonest experimental tumours were those induced in female C3H/HeN mice either chemically, using methylcholanthrene to produce sarcomata [16, 17, 18, 198], or virally, using the mouse mammary tumour virus (MMTV) [250, 290].
In all these animal experiments, using chemically or virally induced tumours, a host versus tumour immune response was detected. After excision of the primary tumour the animal was protected, to some degree, against the establishment of a second inoculum of tumour cells [454, 291] and this protection was lost after splenectomy

17. Billingham *et al* [31] coined the term "adoptive immunity", in their study of skin homografts, to describe the continued function of lymphoid tissue or cells after transfer from an immunized animal to an unimmunized host. Adoptive immunity was shown to be conferred by transferring lymphocytes or splenocytes from a tumour immunized animal to a second host, prior to a tumour challenge, resulting in the rejection of the tumour [101, 467, 198, 141, 17, 18]. The major flaw in these animal studies was that the tumours were either chemically or virally induced, which made them particularly antigenic [311] and when they were compared with truly spontaneous lesions it was notable that, in the latter, there was little evidence of an immune response [346, 189]. Indeed it appeared that the closer conditions in these animal studies simulated the situation in humans the less evidence there was of an immune response to cancer [335].

DELAYED HYPERSENSITIVITY

The limited relevance of animal experiments caused a return to the study of the human immune system and its function in patients with breast carcinoma.
The most commonly used *in vivo* study of the immune

system in breast cancer patients was the test of delayed hypersensitivity. This was performed by injecting intradermally either dinitrochlorobenzene (DNCB), or a similar chemical irritant, to test *de novo* sensitization [279, 302, 349, 393, 447] or an extract of autologous tumour to test specific hypersensitivity [36, 37, 396, 4, 40]. Those who used the first method detected no difference between patients with breast cancer and normal controls nor was any difference seen at different stages of the disease apart from a reduced response in patients who were terminally ill. In these patients, Stein [393] found that tumour dissemination preceded impairment of the patient's immune response, suggesting it might be the cause rather than the result of the impairment.

Studies in which autologous tumour was inoculated, demonstrated a markedly greater response in tumour patients than in those with benign lesions. Patients with stage II disease also had a greater response compared to stage I patients [36, 396, 40]. There was disagreement, however, over whether or not this delayed hypersensitivity reaction represented an advantageous immune reaction associated with a good prognosis [40, 4] or was indicative of a poor prognosis [396].

FUNCTIONAL *IN VITRO* STUDIES

BLASTOGENESIS ASSAYS

One of the standard *in vitro* assays of immunocompetence was the blastogenesis assay. This assessed the ability of the patient's lymphocytes to respond to mitogen stimulation. The commonest mitogen used was phytohaemagglutanin (PHA) and the uptake of tritiated thymidine (3HT) by the cells was measured as an indicator of cell turnover in response to it. The majority of groups found the lymphoproliferative response to PHA to be depressed in breast cancer patients, particularly with advancing disease [461, 462, 302, 393, 447, 212, 272, 271], although two groups found a greater response in breast cancer patients compared with normal controls [128, 201]. Fisher *et al* [128] studied lymph node lymphocytes from both patients and controls and found no difference in the thymidine uptake before stimulation with PHA, but found a wide variation between patients and between nodes within the same patient. This variability may explain some of the conflicting results and the difficulty in interpreting them.

CYTOTOXICITY ASSAYS

The vast majority of these were performed using the chromium release assay whereby tumour cells are cultured in medium containing chromium^{51} which is taken up by them and later released upon destruction or damage of the cells. This damage may be due either the cytotoxic effect of the mononuclear cells under study or to spontaneous cell death and therefore a control preparation is used to assess the background signal. Groups using this method to study tumour infiltrating lymphocytes (TILs) have again produced conflicting results with some showing evidence of specific cytotoxicity [427, 24, 386], while others found TILs to have low levels of cytotoxicity against autologous tumour [444, 15]. These cytotoxicity assays have several limitations. The indirect method of measuring cell death makes it a rather crude assay in which small variations in cytotoxicity are not detectable and the spontaneous cell death among the tumour cells gives a very high control reading. As breast cancer cells cycle slowly and do not grow well in tissue culture it is difficult to obtain large numbers of viable autologous tumour cells containing the chromium.

SURFACE MARKER EXPRESSION

PHENOTYPING

With greater understanding of the immune response and the function of its component cells, interest has developed in identifying the lymphocytes that might be involved in any immune response being mounted by cancer patients, including those with breast cancer. The ability of T lymphocytes to rosette sheep red blood cells (SRBCs) and the presence of surface immunoglobulin on B lymphocytes was utilized in assessing the proportions of these cells present within the tumour, axillary lymph nodes and peripheral blood of breast cancer patients. T cells were found to be the major component of the tumour infiltrate [119, 120, 444]. Of the groups who compared the peripheral blood of normal controls and patients with breast cancer, two found no alteration in the proportion of B and T cells, as measured by these rosetting techniques [116, 171], while two others found a decrease in circulating T lymphocytes with a concomitant increase in circulating B lymphocytes [222, 182]. Eremin *et al* [116, 117] found a similar alteration in the phenotypic proportions within the axillary lymph nodes of breast cancer patients although they found no alteration in the circulating blood

lymphocytes. Other groups found no difference in the circulating lymphocyte proportions between patients with breast carcinoma and normal controls [171, 192].

Tsakraklides *et al* [430] used the rosetting method to compare the proportion of T and B lymphocytes in the lymph nodes of patients with stage I and stage II breast cancer and found an increase in the B cell population in stage II disease.

Anti-sera against T and B lymphocytes were also used to phenotype cells. Two studies used this method to study the tumour infiltrating lymphocytes (TILs) and also found T cells to predominate [374, 380]. At this time it was not possible to subdivide the T lymphocytes into the functional helper or suppressor/cytotoxic subsets.

Since the development of monoclonal antibodies to the surface markers of cell phenotype [-45], it has become possible to compare the type of cells making up any given lymphocyte population in both normal subjects and cancer patients.

This new technology has been applied extensively to the study of the TILs but little work has been done on the peripheral blood or lymph node lymphocytes of breast cancer patients. The studies of the tumour infiltrate have confirmed that this consists largely of T lymphocytes but there is controversy over the further classification of these into subsets. While some

studies show a predominance of CD4+ helper T cells [463, 164, 193, 25, 7, 435, 233, 15], others show a predominence of CD8+ suppressor/cytotoxic T cells [159, 29, 362, 204, 261, 460, 410, 476, 30, 24, 300].

One group compared the proportion of T and B lymphocytes in lymph nodes from breast cancer patients and from patients with non-malignant disease [50]. In the latter case, the nodes were excised because of hypertrophy or infection and cannot truly be considered as controls.

Two groups used flow cytometry to compare the peripheral blood and lymph node lymphocytes (LNLs) of patients with breast cancer but did not include any normal controls and when they found a large CD4+ helper T cell population in the nodes were unable to clarify whether this was due to the lymphocyte source or to the presence of malignant disease [289, 300].

Only two groups have compared the phenotypic proportions of normal lymph nodes with those excised from patients with breast cancer. Khuri *et al* [224] used immunohistochemical techniques and found an increase in the CD8+ T cell population in the nodes from breast cancer patients while Mantovani *et al* [272], using single colour flow cytometry, found no alteration in the phenotypic proportions. These studies were small, with each containing fewer than ten patients.

Four studies have been carried out comparing the

phenotypic composition of peripheral blood lymphocytes (PBLs) from normal controls and patients with breast cancer [265, 260, 325, 272]. The last two of these studies, utilised flow cytometry to study the phenotypic and activation markers. Pattanapanyasat *et al* [325] found a greater proportion of CD8+ T lymphocytes in the blood of breast cancer patients while the others found no phenotypic alterations in early disease. McCluskey *et al* [265], however, did find a marked decrease in the proportion of circulating CD4+ helper T cells in patients with advanced breast cancer.

ACTIVATION MARKERS

Some groups have also studied the activation markers present on the surface of lymphocytes in patients with breast carcinoma. Studying TILs, Rowe & Beverley [367], Lwin *et al* [261] and Ben-Ezra & Sheibani [25] found HLA DR on a fairly large proportion of the lymphocytes within the tumours while Whiteside *et al* [459, 460] found very few HLA DR bearing cells. Lwin *et al* [261] also found Tac, the 55Kd component of the interleukin 2 receptor (IL-2R) on 30% of TILs while other groups found few cells with this receptor [459, 460, 410, 25].
Only one group compared the activation marker expression

of (LNLs) in breast cancer patients and control subjects. Mantovani *et al* [272] found more LNLs from patients with stage I disease to be carrying HLA DR than from control patients, while fewer lymphocytes from patients with stage II disease carried this marker. These differences, however, did not achieve statistical significance. This group did not study the receptors for IL-2 or transferrin (Trf), nor the expression of surface immunoglobulin G (IgG) on lymph node B lymphocytes.

Pattanapanyasat *et al* [325] and Mantovani *et al* [272] studied the expression of HLA DR on the peripheral blood lymphocytes of breast cancer patients and controls and found a slight increase in the number of HLA DR bearing cells in the breast cancer patients. Pattanapanyasat *et al* [325] also studied the receptors for Il-2 and transferrin but found no difference between the cancer patients and controls.

Most of the studies of TILs were made using immunohistochemical methods with a monoclonal primary antibody to the phenotypic surface marker and subsequent enzyme staining, predominantly peroxidase. The later studies used immunofluorescent microscopy with directly labelled monoclonal antibodies while the most recent have used two colour immunofluorescent monoclonal antibodies and flow cytometry.

The use of flow cytometry to study the membrane bound markers, of phenotype or activation, on TILs has been applied to the study of other tumours such as malignant skin melanoma [209, 23], eye melanoma [108], ovarian carcinoma [186], hepatic tumours [407], oesophageal carcinoma [406], and a selection of solid tumours [276, 15]. Although these last two groups did include some breast tumours, their numbers were fairly small and they did not include activation markers in their studies.

By virtue of its ability to collect data on up to 10,000 cells, flow cytometry can make more statistically secure observations without introducing observer error. The use of propidium iodide to exclude dead cells from analysis, avoids non-specific cytoplasmic staining which can be mistaken for membrane staining in cells with a large nucleus and little cytoplasm; such as breast carcinoma cells.

A considerable advantage of flow cytometry is the ability to carry out accurate double staining experiments, allowing both the avoidance of cells registering in more than one phenotypic subgroup and the detection of surface activation markers as carried by different cell phenotypes.

An advantage of immunohistochemistry, which is lost in flow cytometry, is that the relationship of TILs to the overall tumour architecture can be studied.

PRESENT STUDY

In this study we analysed the lymphocytes from the primary tumours of 31 patients, the axillary lymph nodes of 40 patients and the peripheral blood of 39 patients with regard to the proportions of CD4+ helper T cells, CD8+ suppressor/cytotoxic T cells, and B cells. We also looked at the proportion of each cell type carrying the activation marker HLA DR, the receptors for interleukin 2 and transferrin and, in the case of the B lymphocytes, surface membrane IgG. These parameters were also studied on the lymph node and peripheral blood lymphocytes of 7 control subjects undergoing vascular surgery or organ donation.

The tumour cells themselves were stained for the expression of the class I and class II major histocompatibility antigens and for membrane bound IgG. The phenotypic proportions and activation status of the tumour infiltrating lymphocytes were correlated with the tumour cell expression of class I and class II MHC antigens and with the common prognostic indicators of tumour stage, histological tumour grade and oestrogen receptor status.

The composition and activation status of the lymph node and peripheral blood lymphocytes were correlated with tumour stage.

METHODS

FLOW CYTOMETER

This study of lymphocytes from the tumour, regional lymph nodes and peripheral blood of breast cancer patients was performed by flow cytometry using a FACScan analyser (Becton-Dickinson, Cowley, Oxford). This cytometer does not have a cell sorting capacity but is purely for the analysis of cell suspensions with regard to cell size, granularity and the presence of fluorochromes on or within the cell. It utilises a 15mW Argon ion gas laser because 15mW is the level at which the excitation of fluorochromes, conjugated to monoclonal antibodies, reaches a plateau and higher energy input brings little increase in the energy emission from the fluorochrome. Due to its low power, the laser can be air cooled and this allows its use as a bench top instrument by the investigator involved in the study rather than requiring both a purpose built room and a committed operator. The laser is set at a fixed wavelength of 488nm and therefore excites only fluorochromes in the red-green spectrum. While this limits the fluorochromes that can be used it makes the machine more reliable and easier to operate.

The cytometer measures and records up to five parameters on each cell. The forward scatter of the laser light (FSC) which, like a shadow, is related to cell size, the 90° side scatter (SSC), which is the light reflected from the cell and is related to granularity and the binding of up to three fluorochromes. The fluorochromes used in this study were fluorescein isothiocyanate (FITC) which emits in the green waveband from 480nm to 600nm, phycoerythrin (PE) which emits in the orange-red waveband from 550nm to 640nm and propidium iodide (PI) which emits at the strong red end of the spectrum from 550nm to 700nm.

FLUIDICS

The function of the fluidics system is to provide a single file procession of cells through the viewing orifice of the flow cell. This is achieved by creating a laminar flow and driving the cells into a single stream (Fig. 2).

The cell suspension under study is prepared as described later, taking care to avoid contamination by cell debris which might clog the machine. The cell suspension (about 0.5ml) is placed in a cytometer tube (Falcon 2052) and pushed over the sample pipette which is held

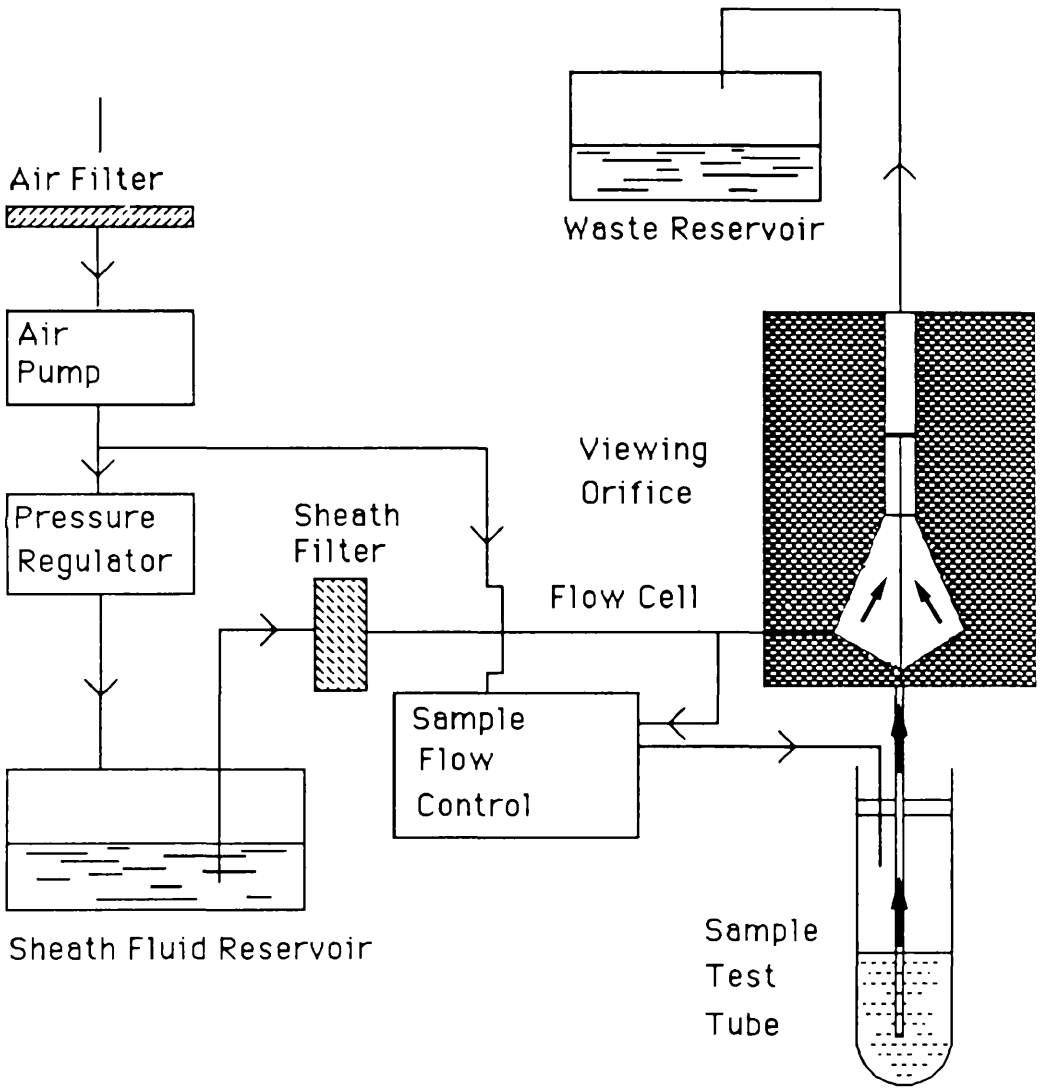


Fig. 2 A box diagram of the flow cytometer fluidics system which produces a laminar flow within the flow cell and forces a stream of cells into the viewing orifice in single file.

in place by a rubber grommet to give an airtight seal. The pressure within the tube is increased slightly, by pumping in air, and this propels the sample up into a conical chamber in the flow cell assembly. Here it is forced into a laminar flow by a stream of isotonic sheath fluid driven under pressure from a tank maintained at 4.5PSI by an air pump. The laminar flow causes the cells to form a single stream as they leave the conical chamber and enter the flow cell where they are exposed to the argon-ion laser and their behaviour in the laser light is recorded. The flow cell of the FACScan analyser is a rectangular quartz cuvette with a large internal cross-section of 180u by 430u to avoid blockage by cell clumps or debris. After this the flow of sheath fluid carries the cell sample round to a waste tank. The stream of cells through the flow cell is controlled by the speed of the sheath fluid. A flow rate of 60ul/minute is used for the immunofluorescence of cell-bound monoclonal antibodies while DNA studies are carried out at the slower rate of 12ul/minute.

OPTICS

The argon ion laser beam is first passed through a prismatic refracting beam expander and then through a

lens, to produce a converging elliptical beam, 20u vertically by 60u horizontally. This is focused on the stream of cells in the flow chamber by a steering plate and the behaviour of the cells within the laser light is recorded (Fig. 3).

The forward passage of the beam is scattered by each cell and this increases with size in a similar way to the shadow of an object in normal, radiant light. This forward scatter (FSC) is recorded on a silicon photodiode lying in the path of the laser on the opposite side of the flow chamber.

The reflection of the laser light from the cell increases with granularity and this reflected light is collected, along with the fluorescence, through a condenser lens set at 90° to the laser beam. This is a bi-convex lens which converts the scattered light and fluorescence into parallel light.

The reflected light or side scatter (SSC) and the green fluorescence (FL1) are separated from the red fluorescence by a 22.5° dichroic mirror with a 560nm edge which reflects longer wavelengths and transmits shorter wavelengths. The red fluorescence is reflected by this mirror while the green fluorescence and side scattered light pass through it to be separated from each other by a Brewster window. This is a quartz plate set at an angle between 53° and 57° which reflects the

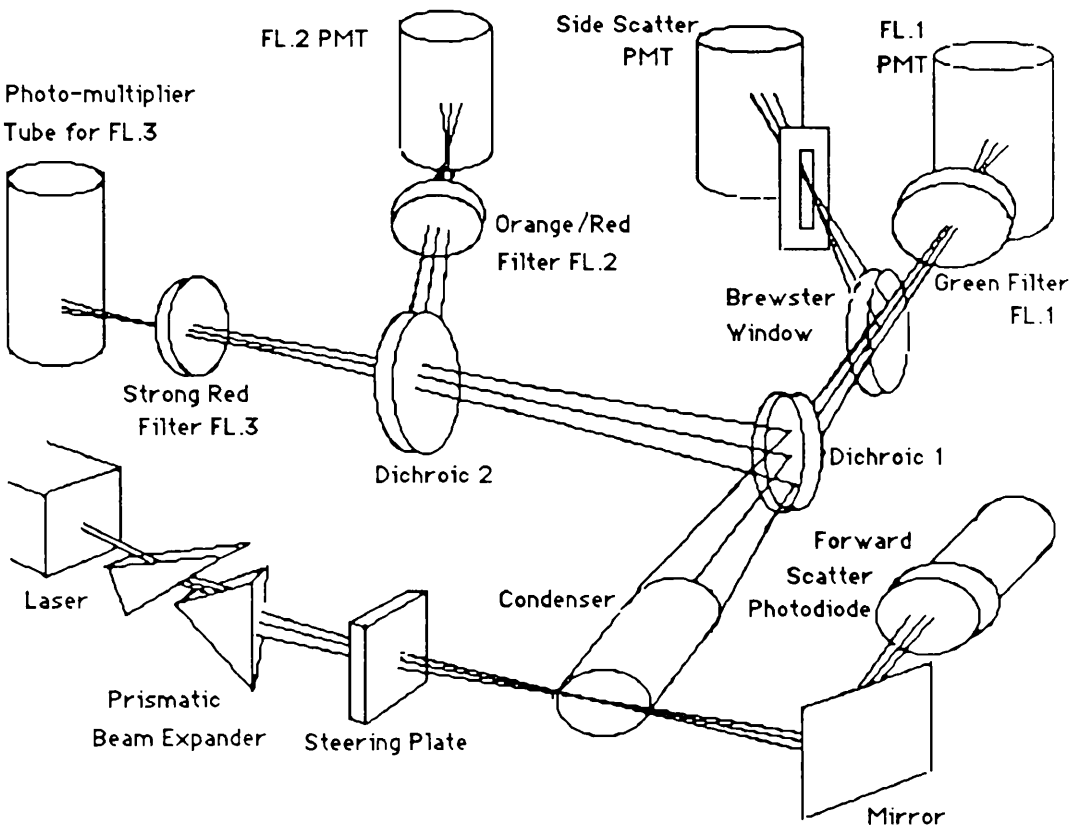


Fig. 3 A diagram of the optical system within the flow cytometer which uses a series of mirrors and filters to separate the fluorescent emission, from the cell, into its green, orange-red and strong red components.

side scattered light because it is polarized, while allowing the green fluorescence to pass through without loss.

The green fluorescence then continues through a band pass filter with a wavelength of 530nm and width 30nm. The red fluorescence, after being reflected by the first dichroic mirror, is separated into strong-red (FL3) and orange-red (FL2) wavebands by a second dichroic mirror, this time set at 45° with a 640nm edge. The strong-red fluorescence passes through this and through a long pass filter of wavelength 640nm while the orange-red fluorescence is reflected and passes through a band pass filter with a wavelength of 585nm and width 42nm.

DETECTION AND DISPLAY

The side scatter (SSC) and fluorescent emissions of the three fluorochromes are each detected by separate photoelectric multipliers.

The photons enter the photoelectric cell and each photon excites one electron which is reflected backwards and forwards off alternate parallel cathode plates. These carry an electric charge to accelerate the electrons and so each electron that strikes the cathode causes the emission of several more, thus leading to a great

amplification of the original signal.

It can be seen from the emission wavebands of each fluorochrome that there is considerable spectral overlap between them (Fig. 4). This is of particular importance between FITC (FL1) and PE (FL2) as these are the fluorochromes conjugated to the cell surface marker antibodies and are therefore among the parameters being measured. This overlap of the FITC signal into the PE channel and vice versa is therefore compensated electronically, as is that between PE and PI.

The final signal for each parameter is in the form of an analog electronic pulse which is converted to digital data by an analog/digital converter to allow storage and processing by the computer system. The data management system is based on the HP310 computer (Hewlett-Packard) and this is connected to the cytometer by a parallel interface (GP10). The software program utilised in this study was FACScan Research which allows the collection and storage of raw data from all five parameters in list mode and display and analysis on a screen with 1024 channel resolution.

The limit of sensitivity is 1000 FITC equivalent molecules per cell and, as the amount of fluorescence borne on the surface of the cells varies widely, the resultant range is displayed on a four decade log scale to allow easier measurement and analysis.

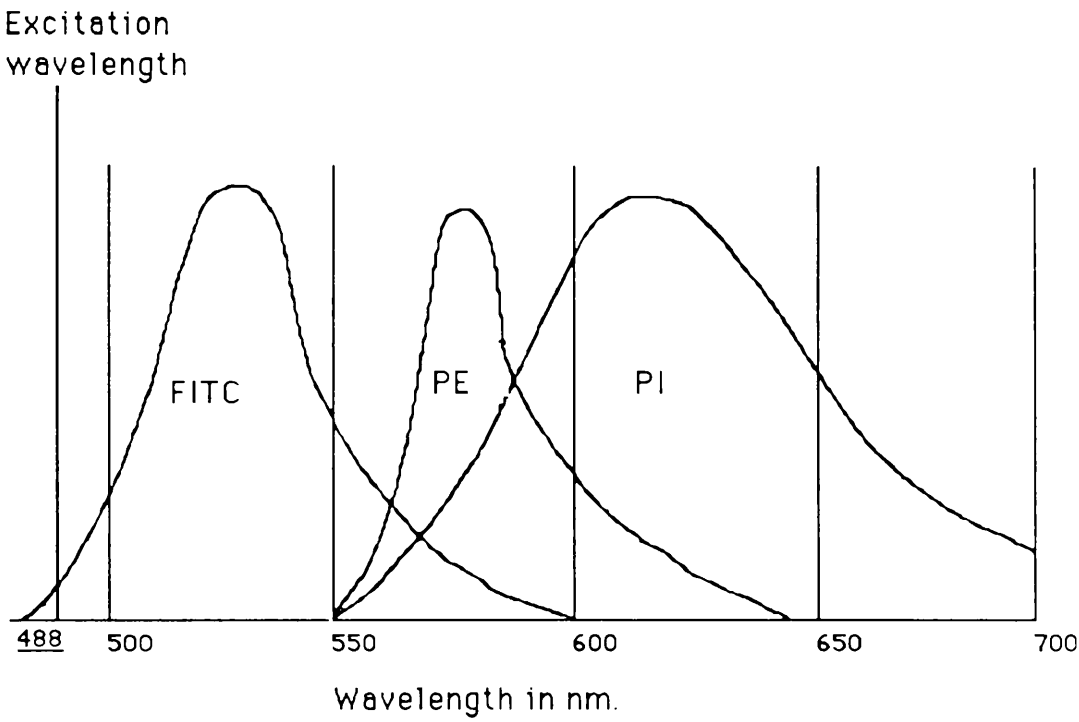


Fig. 4 Showing the overlap of the emission spectra of the fluorochromes used in this study. This is corrected for by an electronic compensation.

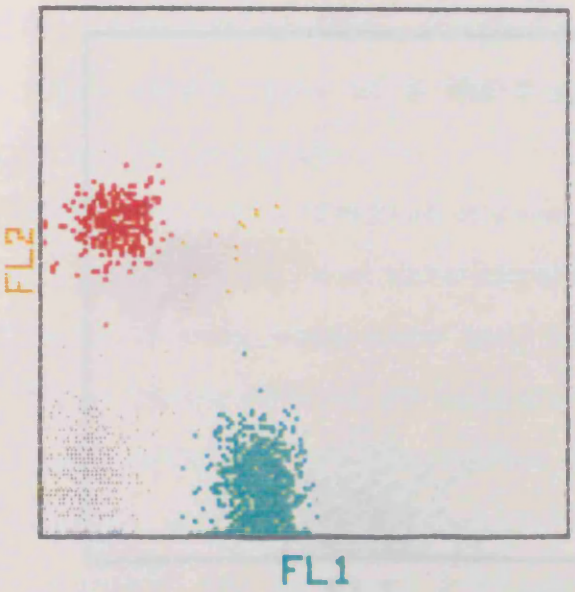
The preparation of cells for the flow cytometer and analysis of the data obtained are outlined later.

SURFACE MARKERS

PHENOTYPIC MARKERS

The lymphocytes were identified phenotypically using combined "Simultest" preparations of two antibodies, one conjugated with PE, the other with FITC (Becton Dickinson, Oxford). This allowed the identification of two phenotypes at the same time with those outwith these subsets remaining unstained at the origin (Fig. 5).
The lymphocytes were phenotyped as B or T lymphocytes using PE conjugated Leu 12 antibody to the CD19 antigen on the surface of B cells and FITC conjugated Leu 4 to the CD3 antigen on T cells. The T cells were further phenotyped into their subsets using Leu 2a, conjugated with PE, to the CD8 antigen to identify the suppressor/cytotoxic T cells. It is not yet possible to separate this subgroup further into suppressor cells and cytotoxic cells.
The Leu 3a antibody, conjugated with FITC, was used to identify the CD4 antigen on the helper T cells. As yet there there does not appear to be a clear delineation,

CD4+ Helper T cells



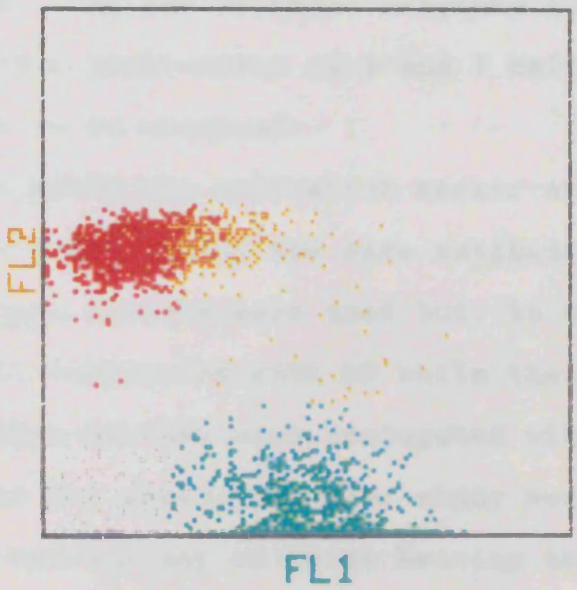
B cells

CD8+ T cells
(suppressor/cytotoxic)

Fig. 5 A coloured dot-plot showing the use of dual immunofluorescence to analyse the phenotypic proportions of a lymphocyte population. In this sample of lymph node lymphocytes, the CD4+ helper T cells are stained with a PE conjugated antibody and show up as red, the CD8+ suppressor/cytotoxic T cells, stained with FITC, emit green fluorescence while the unstained B cells remain at the origin.

CD4+ T cells

CD4+ T cells also bearing HLA DR



Cells bearing neither marker

Non-CD4+ cells bearing HLA DR (B cells and activated CD8+ T cells)

Fig. 6 A colored dot-plot showing the use of dual immunofluorescence to measure the proportion of activated cells within any phenotypic subgroup. In this sample, PE conjugated Leu 3a antibody is used to identify the CD4+ helper T cells and those also bearing HLA DR are identified using a FITC conjugated antibody. In the coloured dot-plot, the cells carrying both red and green fluorochromes show up as yellow.

as is seen in the murine system, between CD4 T cells which provide help for B cells and those which stimulate T cells. The use of these reagents allowed the phenotypic proportions of B and T cells and the T cell subsets to be measured.

When studying the activation marker expression within each cell population the same antibodies to the cell phenotypic markers were used but, in this case, they were all conjugated with PE while the antibodies to the activation markers were conjugated with FITC. Thus, cells of the phenotype under study would be stained orange-red and any of those bearing the activation marker of interest would also be stained green by the FITC conjugated antibody. On a coloured screen this mixing of green and red results in yellow (Fig. 6).

ACTIVATION MARKERS

Class I major histocompatibility complex

To avoid confusion with tumour antigen we will refer to the major histocompatibility (MHC) "complex" rather than MHC "antigen".

The class I MHC complex is made up of two polypeptide chains. One of these traverses the cell membrane, is glycosylated with a molecular weight of 45,000 and has

three domains, alpha 1, alpha 2 and alpha 3. The other, the beta-2 microglobulin, is not transmembranous, is non-glycosylated and has a moleclular weight of 12,000. The alpha 3 domain and beta-2 microglobulin form the base of the complex near to the cell membrane while the antigen presenting groove is constructed from the alpha 1 and alpha 2 domains on the heavy chain (Fig. 7). The walls of the groove are formed from the side chains of the alpha helices while the base is made up of the side chains of the central beta strands on these alpha domains [33] (Fig. 8).

CD8+ suppressor/cytotoxic T cells can only recognize and bind antigen in combination with this complex. This combined recognition prevents the antigen receptors on CD8+ T cells from being blocked by free circulating antigen, such as virus, and ensures the killing of infected cells [165, 104, 208, 184] (Fig. 9).

While benign breast lesions and medullary tumours appear to have fairly uniform cell expression of the class I MHC molecule [298, 463], some groups have demonstrated a lack of class I MHC complexes on the cells of many breast tumours [143, 339, 181] and as the immune system, and in particular the CD8+ cells, can only recognize antigen in the presence of this complex, its lack might prevent the recognition of tumour antigen. We therefore studied the expression of this marker on the tumour

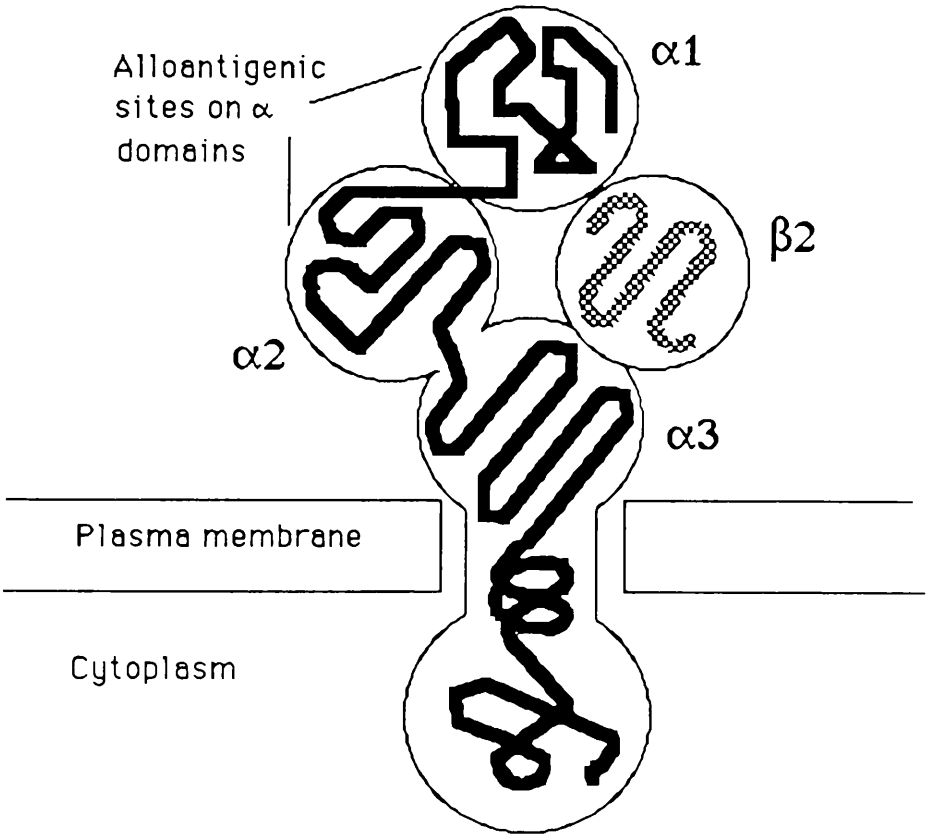


Fig. 7 A schematic drawing of the structure of the Class I major histocompatibility antigen showing the three α domains and the β_2 microglobulin.

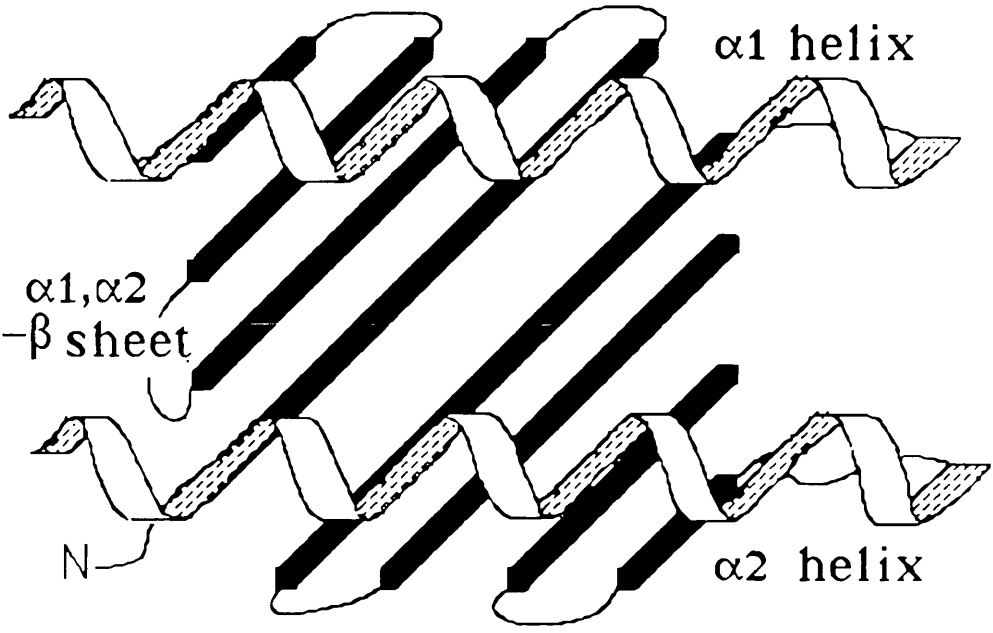


Fig. 8 The antigen presentation groove, within which antigen is held, is approximately 10Å by 25Å. The walls are the side chains of the α helices while the floor formed by the side chains of the β sheet between the two α domains.

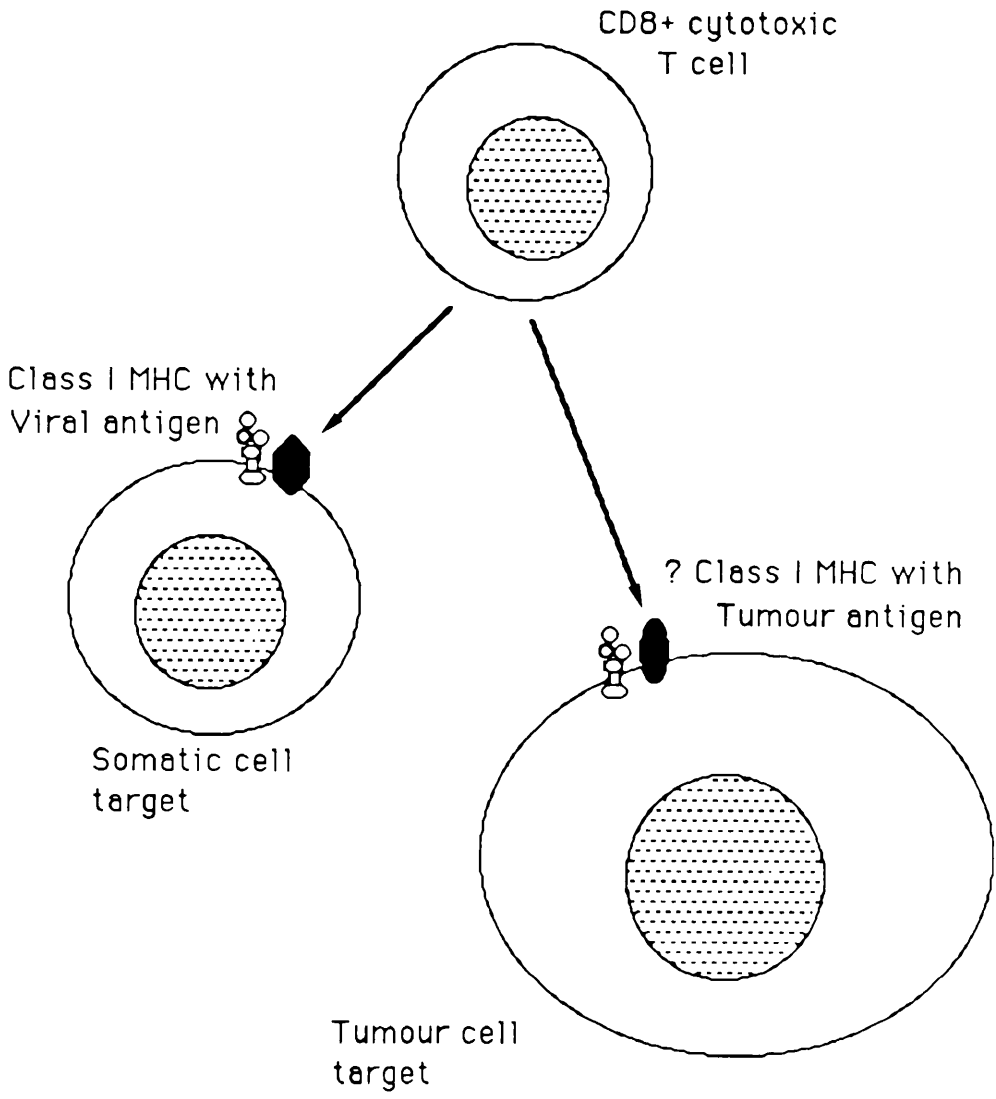


Fig. 9 CD8+ cytotoxic T cells recognize foreign antigen presented by the Class I MHC structure on somatic cells. It may be that they can recognize tumour antigen in a similar fashion on tumour cells which are expressing Class I.

cells and correlated it with the degree and type of immune reaction found within the tumours. This marker was detected using the W6-32 antibody which was used with a second goat anti-mouse antibody conjugated to FITC.

HLA DR

HLA DR is the predominant antigen of the class II MHC complex. The structure of this complex is less well known but does appear to be similar to that of the class I complex except that the four domains are arranged on two polypeptide chains of similar size, both of which are transmembranous in position [60] (Fig. 10).
Antigen is recognized by CD4+ helper T cells when it is presented in conjunction with this MHC complex in a similar fashion to the recognition of antigen, in conjunction with the class I MHC complex, by suppressor/cytotoxic T cells (Fig. 11). The antigen presenting cells include B cells and macrophages and this complex is usually found on these cells [178]. The antigen presenting capability of B cells has been shown to relate directly to the degree of HLA-DR expression on their surface [471].
HLA-DR has also been demonstrated as a T cell activation marker which is absent on resting T cells [338] but is present on T cells stimulated by antigen, in mixed

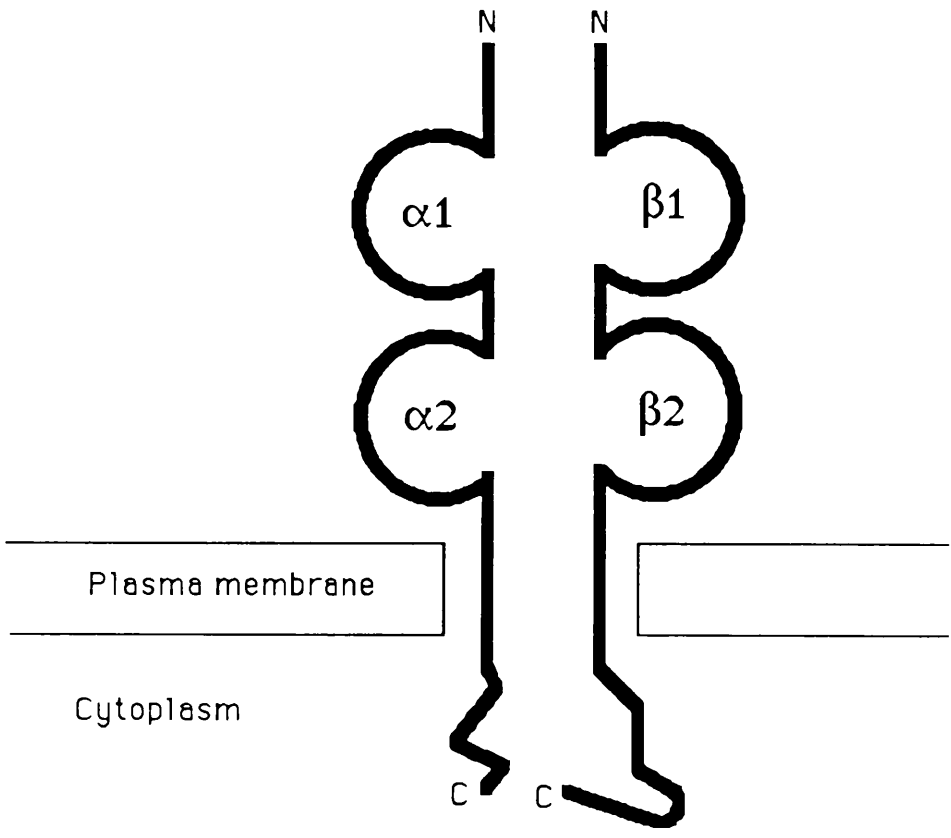


Fig. 10 In the case of the Class II MHC structure, the α and β domains are similar in size and are both transmembranous.

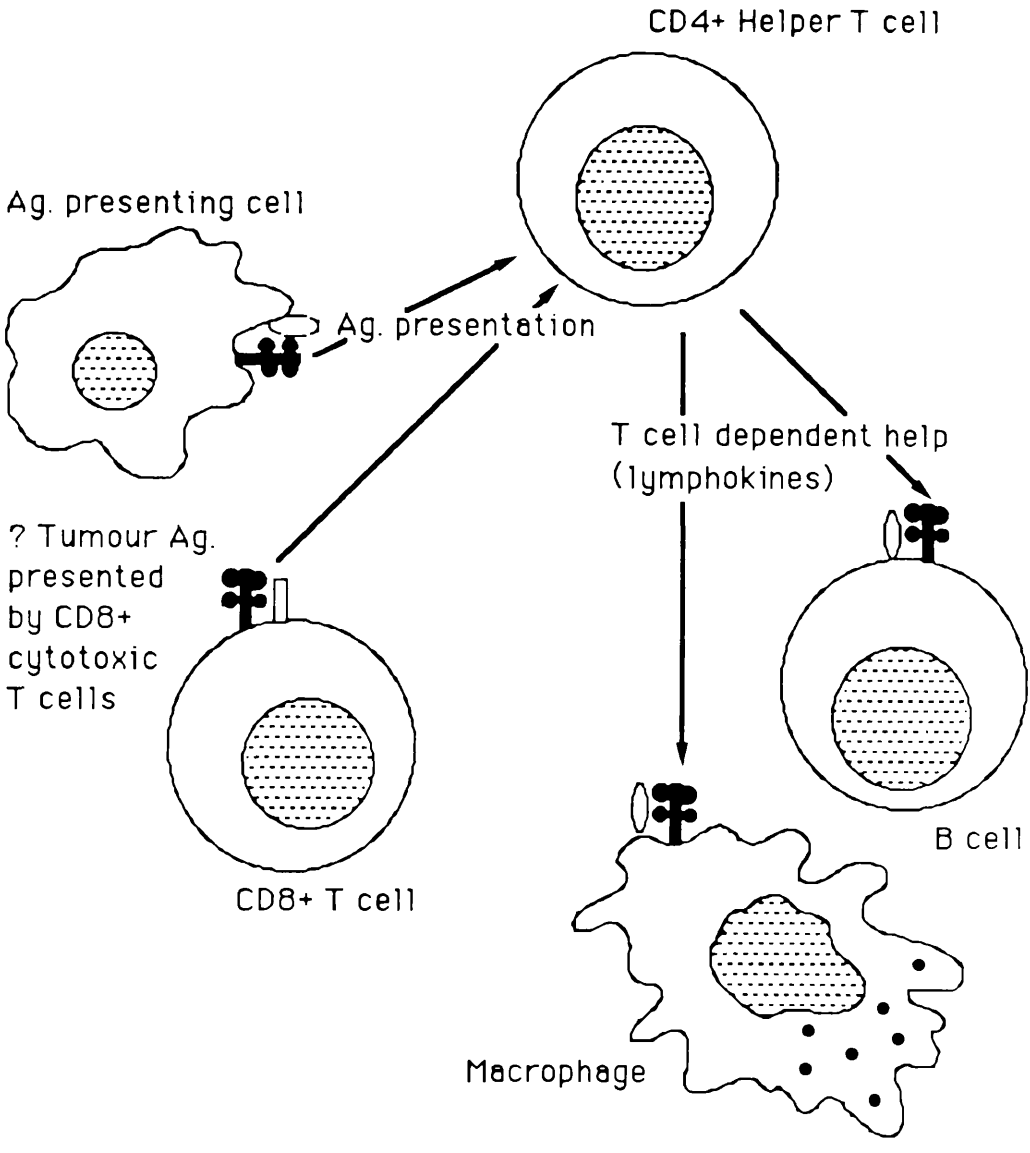


Fig. 11 CD4+ helper T cells are stimulated by antigen presented along with the Class II MHC structure and they, in turn, stimulate macrophages and B cells by secreting lymphokines. Activated CD8+ suppressor/cytotoxic T cells express the Class II structure and may present antigenic material from cells they have destroyed.

lymphocyte cultures [326, 98], or mitogen [234]. Although its exact function on T cells is unknown, there is some evidence to suggest it is involved in antigen presentation as is the case with B cells [338, 326]. Some role in cell communication is also suggested by the number of CD4+ helper T cells which bear this marker and the finding that blocking the class II complex with a specific antibody prevents activated cells from responding to interleukin 1 [322].

In this study, therefore, it was used as a marker of T cell activation along with antigen recognition and presentation.

This complex has also been identified on certain epithelial cells and the murine equivalent has been found on the mammary glands of mice [232]. Studies of renal graft rejection have shown a strong correlation between rejection of the graft and the expression of this complex on both the tubular epithelium of the graft and the circulating T cells [11, 194]. We therefore also studied the expression of this complex on the tumour cells and correlated this with parameters of the intratumoral immune response.

The class II MHC complex was stained with a FITC conjugated antibody to HLA DR from Becton Dickinson.

Interleukin 2 receptor

The receptor for the lymphokine interleukin 2 is composed of two glycoprotein chains, an intermediate affinity alpha chain with a molecular weight of 75,000 which transduces the signal into the cell [433, 414, 347] and a beta chain of low affinity with a molecular weight of 55,000 [436]. When combined, the affinity increases a thousand fold because of kinetic cooperation between the two chains. The 55,000 beta chain binds interleukin 2 briefly and then dissociates, which brings the IL-2 into the plane of the membrane and allows the 75,000 alpha chain to bind it (Fig. 12).

This chain binds and dissociates from IL-2 very slowly and these cooperative kinetics create a very efficient receptor [367]. On antigen stimulated T cells the expression of the low affinity beta chains is 5 to 10 fold greater than that of the intermediate affinity chain. IL-2 binding to high affinity receptors decreases their expression by 50%, but increases the expression of the beta chain 10 to 20 fold [387], producing an excess of beta chains and allowing the number of high affinity receptors to be controlled by the number of alpha chains. This increase in beta chains makes their presence a good guide to the increase in IL-2 receptors after activation, antigen stimulation or in the presence of IL-2.

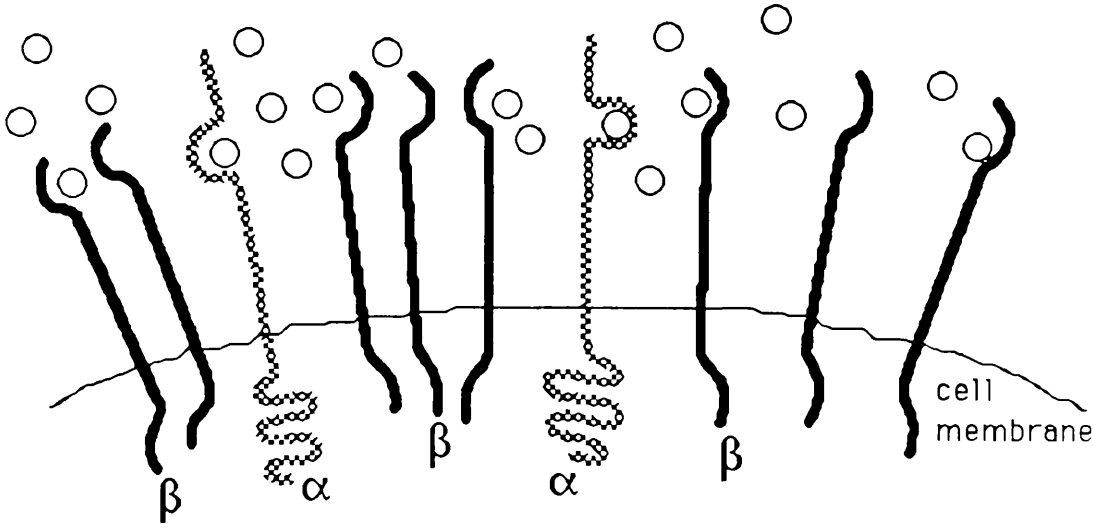


Fig. 12 A schematic representation of the high affinity Interleukin-2 Receptor showing the kinetic cooperation between the two chains. The β chain binds the Interleukin-2 only briefly before releasing it within the plane of the membrane thus making it possible for the β chain to bind it and transduce the signal into the cell

Expression of the interleukin 2 receptor increases after stimulation, by mitogen or antigen, and IL-2 then mediates the movement of the cell from $G1_a$ to $G1_b$ ready to enter the S phase of the cell cycle [28]. The expression of this marker precedes that of the Trf receptor in actively dividing cells.
The anti Tac antibody detects CD-25, an antigenic determinant on the beta chain of the IL-2 receptor and this was therefore used as an activation marker on T lymphocytes. Tac was also used as an activation marker on B cells where it is expressed at about one third of the level of T cell expression [259].
Tac was stained with a FITC conjugated antibody to CD25 from Becton Dickinson.

Transferrin receptor

This receptor consists of a transmembranous glycoprotein with a molecular weight of about 95,000, made up of 2 identical subunits bound by di-sulphide bonds.
Iron in its ferric form binds to transferrin (Trf) which then binds to the Trf receptor. Ferric-transferrin is then endocytosed on the receptor to an acidic, non-lysosomal compartment where the iron is released from the protein and transported across the cell membrane into the cytoplasm. The Trf protein (apo-transferrin) remains on the receptor and is

recycled to the cell surface and released leaving the receptor free again [417] (Fig. 13).

This receptor is poorly expressed on resting and well differentiated cells but strongly in tissues with a high proportion of dividing cells [419, 401] or on mitogen stimulated lymphocytes [398, 146, 149]. The changes in the Trf receptor occur prior to DNA synthesis [81, 58] with the peak just prior to cell division [7-]. This increase in Trf receptors prior to cell division is due to the increased need for intracellular iron, required by the enzyme ribonucleotide reductase which is involved in RNA transcription.

These receptors appear on cells passing from late G1 phase through S phase to cell division after cell stimulation, the appearance of IL-2 receptors [301], and stimulation by IL-2 [19].

We studied this marker on the lymphocytes of patients with breast cancer and normal subjects to see if there was increased turnover of these cells in the cancer patients. This marker was stained using a FITC conjugated anti-CD71 monoclonal antibody from Becton Dickinson.

Membrane immunoglobulin G

Immunoglobulin M (IgM) appears in the initial stages of a primary humoral immune response but is poly-valent and

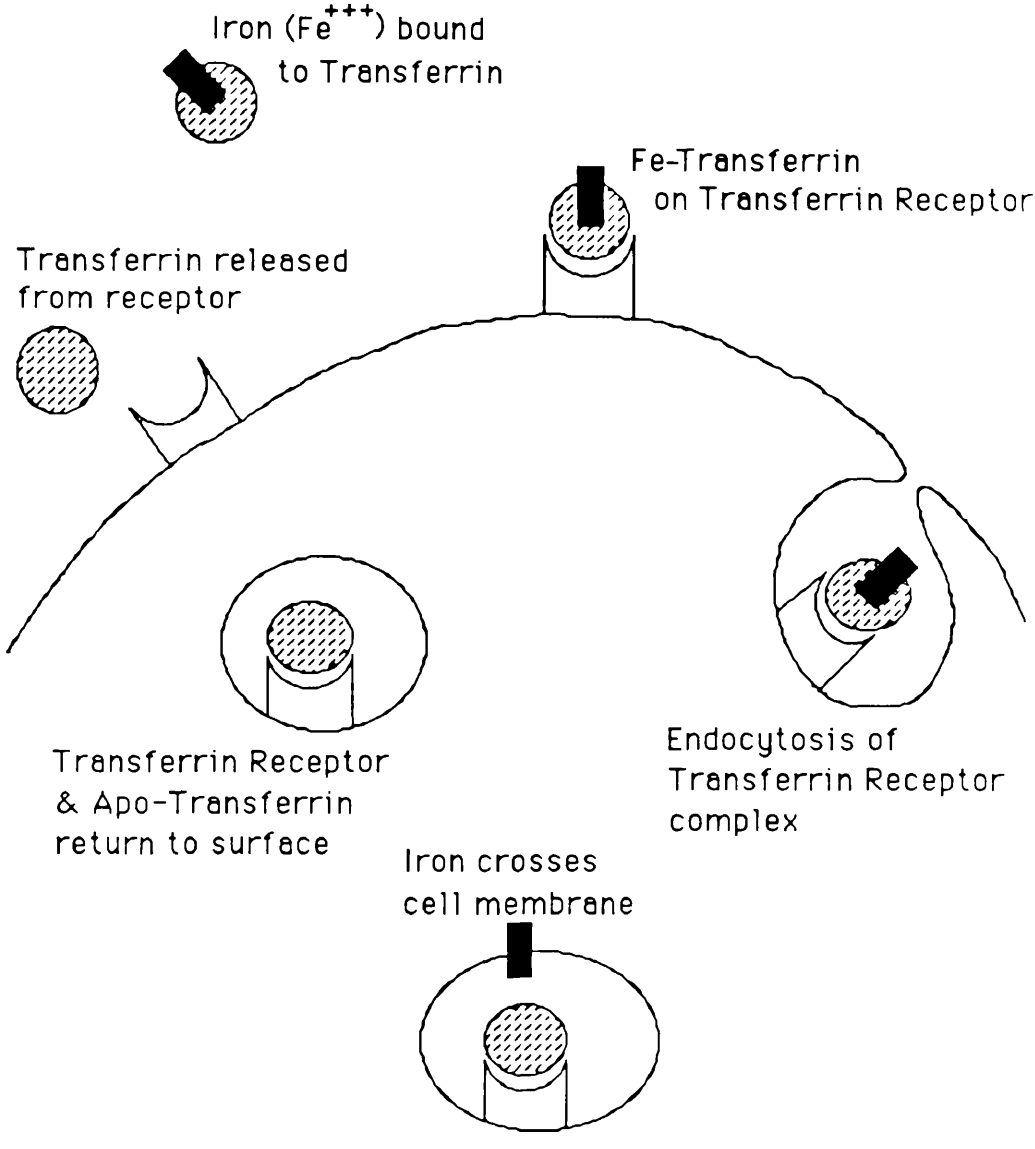


Fig. 13 Ferric iron, bound to Transferrin, is carried into the cell by endocytosis of the Transferrin Receptor. After tranfer of the iron accross the true cell membrane, the Receptor and Transferrin are re-cycled to the surface.

of fairly low affinity. After isotype switching and affinity maturation IgG is produced. This bi-valent immunoglobulin is of much higher affinity and it is this immunoglobulin class which appears during the mature secondary immune response [351] (Fig. 14). Production of IgG may also continue in the presence of chronic antigen although in some cases, when patients develop hyperimmunity, the isotype switching continues until IgE is produced. We therefore used the expression of surface membrane IgG as an indicator of a mature humoral immune response. This was stained with a Dako rabbit $F(ab)_2$ anti-human IgG, conjugated with FITC. Artifactual detection of monocytes binding IgG to their Fc receptors [464] was avoided by the double staining of B lymphocytes with the PE conjugated anti-CD19 antibody.

PATIENTS AND SAMPLES

Samples of primary tumour, axillary lymph nodes and peripheral blood, or all three, were obtained from 46 patients undergoing definitive surgery for breast carcinoma and seven control patients, five of whom were undergoing aortic bifurcation graft surgery while two were cadaveric organ donors. Two of the control subjects were female and five were male, with an average

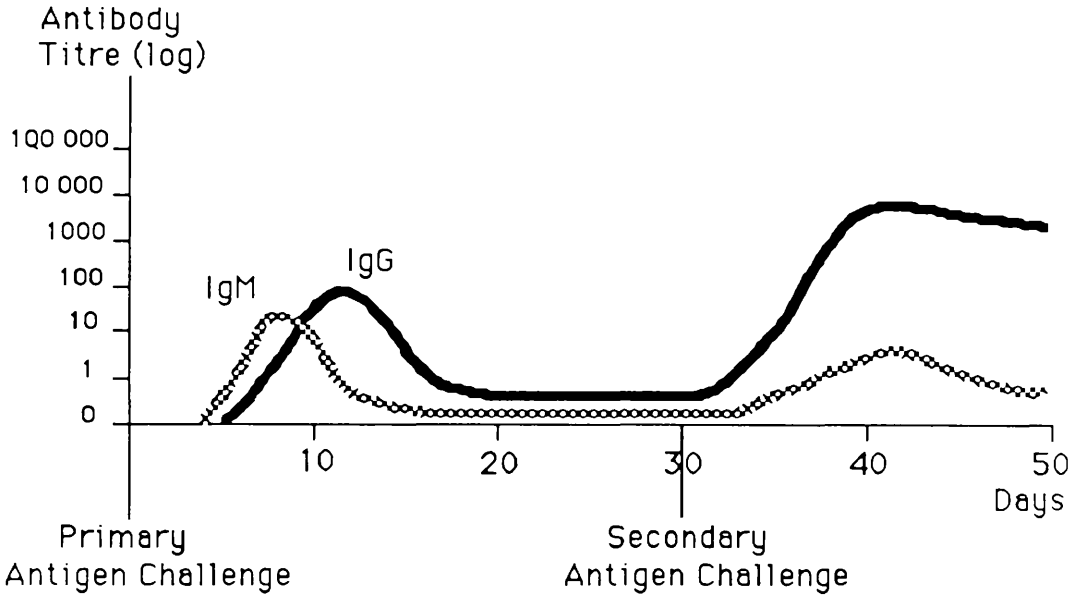


Fig. 14 While IgM is the immunoglobulin produced shortly after initial antigen exposure, IgG secretion follows within a few days and it is this latter which is the major immunoglobulin secreted during a secondary immune response.

age of 48 years, while all of the breast cancer patients were female and the average age was 61 years.

None of the patients with breast cancer had received preoperative cytotoxic, endocrine or radiotherapy and there was no evidence, in either group, of concomitant disease or therapy which might cause immune stimulation or depression.

The samples were collected aseptically in the operating theatre and later processed in a laminar flow hood. Providing this would not compromise the amount of tissue available for routine investigations, a central slice, representative of the tumour as a whole, was taken. The remainder was sent to the Western Infirmary department of pathology for routine histology and grading [47], and to the biochemistry department for oestrogen receptor assay by the ligand binding method [252]. The lymph nodes collected were bisected and while one half was processed for this study, the other was also sent for routine histological staging. 10ml of peripheral venous blood was taken during the operative procedure and collected in potassium ethylene diamine tetra-acetic acid (EDTA) tubes.

CELL HARVESTING

BREAST TUMOUR SAMPLES

Tumour samples were collected from 31 patients. 29 of these subsequently proved to have invasive ductal breast carcinoma while two had lobular breast carcinoma. The tumour samples were washed in a petri dish containing RPMI 1640 medium and, after trimming away any fat, the sample was transferred to another petri dish and mechanically disaggregated using a scalpel and needle to tease apart the tumour tissue and release the cells into the medium. The spilled cells were pipetted off, washed, resuspended in freezing medium consisting of 10% dimethylsulphoxide (DMSO) and 90% foetal calf serum (FCS) and stored in liquid nitrogen.

No attempt was made to separate the tumour infiltrating lymphocytes (TILs) from the tumour cells as many would be lost and this might distort the proportions of various mononuclear cells present within the infiltrate. Surface marker analysis was performed only on cells spilled mechanically from the tumours because a lengthy incubation in collagenase might allow changes to occur in the membrane markers [459, 277] and preparation was carried out shortly after receipt of the samples to minimise turnover of the membrane receptors.

A similar method was applied to 6 normal breast tissue samples obtained at the time of reduction mammoplasty but few cells were harvested and very few lymphocytes seen. It was not practicable to analyse so few cells by flow cytometry and reasonable cell numbers could only be achieved after at least two weeks in culture. This was not comparable with fresh samples and therefore, no control lymphocytes were available from normal breast tissue for comparison with the TILs. Benign lesions could not be used as controls in this study because all of the lesion must be submitted for frozen section histology to exclude malignant disease and, while this would have allowed study of these lesions using immunohistochemical techniques, it meant there was no fresh tissue available for flow cytometric analysis. This is an advantage which remains with immunohistochemical analysis which can be performed on sections cut from the original frozen block.

LYMPH NODE LYMPHOCYTES

Lymph node samples were obtained from 40 patients undergoing definitive surgery for breast cancer which included excision sampling of the ipsilateral axillary lymph nodes. Iliac lymph nodes were collected from 7

normal controls consisting of 2 cadaveric kidney donors and 5 patients undergoing aortic bifurcation vascular surgery. The cells were released from the nodes by teasing them apart with a scalpel and needle. The lymphocytes were isolated from the lymph node suspension by layering this over an equal volume of Ficoll Hypaque (Pharmacia) and centrifuging at 500 G for 20 minutes. The lymphocytes were removed from the interface by careful pipetting, minimising the contamination by Ficoll or RPMI medium [57]. The lymphocytes, thus obtained, were then washed, resuspended in freezing medium and stored in liquid nitrogen until they could be analysed on the flow cytometer.
All samples were processed as quickly as possible to avoid turnover or any major alteration in the membrane receptors.

PERIPHERAL BLOOD

10ml of peripheral venous blood was obtained from 39 patients with breast carcinoma and 7 control patients, during surgery, and collected in potassium EDTA tubes. The blood was diluted with 10ml of RPMI medium and this cell suspension was gently layered over 10ml of Ficoll Hypaque (Pharmacia) and the lymphocytes obtained by

density gradient separation. These lymphocytes were also washed and resuspended in freezing medium to be stored in liquid nitrogen for later analysis on the flow cytometer.

FLOW CYTOMETRY

STAINING OF CELLS

The basic method of cell staining and preparation was the same regardless of whether the lymphocyte sample was from the primary tumour, lymph nodes or peripheral blood.

The cell samples were quickly thawed, in a water bath at 37°C, to avoid damage by ice crystal formation within the cells. The cells were washed twice in filtered phosphate buffered saline (PBS) and resuspended at a cell density of approximately 2 X 10^7 cells/ml. 50ul of this cell suspension was placed into each of fourteen flow cytometry test tubes (Falcon 2052) and monoclonal antibodies, to the phenotypic and activation membrane markers on the cells, were then added (Table 1). Apart from the W6 32 antibody to the class I MHC antigen, these antibodies were directly conjugated with fluoroisothiocyanate (FITC) or phycoerythrin (PE) which

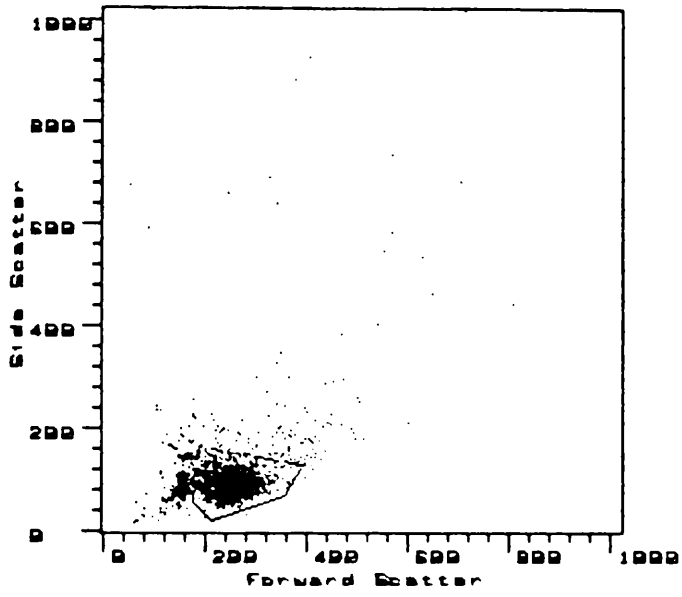
TABLE 1. Monoclonal antibodies used in this study.

Antibody	Predominant Reactivity.
IgG1-FITC + IgG2a-PE	Irrelevant antibody as control.
Leucogate (Anti-CD 45 + Anti-CD14)	Analysis of leucocyte subpopulations (lymphocytes, monocytes, neutrophils).
Simultest (Phenotype analysis)	
Anti-Leu 2a PE	Suppressor/cytotoxic T lymphocytes.
Anti-Leu 3a FITC	Helper T lymphocytes.
Anti-Leu 4 FITC	T lymphocytes.
Anti-Leu 12 PE	B lymphocytes.
Activation analysis	
Anti-Leu 2a PE	Suppressor/cytotoxic T lymphocytes.
Anti-Leu 3a PE	Helper T lymphocytes.
Anti-Leu 12 PE	B lymphocytes.
Anti-HLA DR FITC	HLA DR, the Class II MHC antigen.
Anti -CD-25 FITC	Interleukin 2 receptor, (Tac).
Anti-Transferrin receptor FITC	Transferrin receptor.
Anti-Human IgG FITC	Membrane bound IgG on B lymphocytes.
Tumour cell analysis	
W6 32 antibody with goat anti-mouse 2y antibody	Tumour cell expression of the Class I major histocompatibility antigen.
Anti-HLA DR FITC	Tumour expression of the Class II MHC antigen.
Anti-Human IgG FITC	IgG bound to the membrane of the tumour cells.

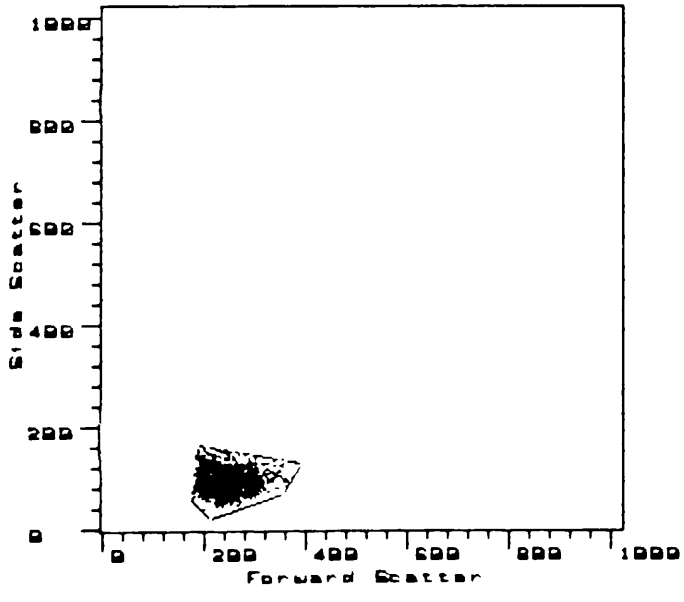
emit green and orange-red fluorescence respectively and were supplied by Becton Dickinson, Cowley, Oxford. After addition of the antibodies, the tubes were agitated on a vortex and incubated for twenty minutes. This was carried out in the dark to prevent bleaching of the PE. To avoid capping and internalisation of the antibody/antigen complexes, 0.02% w/v sodium azide was added and the tubes were kept on ice. After incubation with the fluorescent antibodies, the cells were washed and resuspended in filtered PBS. Propidium iodide was added to each tube to a final concentration of 2ug/ml, to allow the identification and exclusion of dead cells from the analysis. The cell samples were then run through the flow cytometer and data on 5000 live cells collected.

LIVE COLLECTION GATES

The size and granularity of cells affect the forward and side scatter of laser light from them and these properties can be used to differentiate between lymphocytes, monocytes and neutrophils. In this study a gate was drawn around the lymphocyte populatiion to exclude the other cell types and collect data only on lymphocytes (Fig. 15).



Bit-map collection gate around lymphocytes based on FSC and SSC.



Turning gate on excludes other cell types from analysis

Fig. 15 The forward scatter (FSC) and side scatter (SSC) of laser light from lymphocytes, is used to identify them and a collection gate, based on these properties, excludes other cell types from analysis.

A further gate was set to exclude dead or damaged cells which have strong red fluorescence (FL3) due to the uptake of propidium iodide, through breached membranes, into their nuclei. Using this gate data was collected only on live cells (Fig. 16).

These gates were not applied when the degree of lymphocytic infiltrate within the tumours was being studied as they would have altered the cell proportions considerably. Leucogate, a combined antibody preparation which differentially stains lymphocytes, monocytes and neutrophils, was used to measure the degree of lymphocytic infiltrate within the tumours and also to assess the effectiveness of the lymphocyte gate (Fig. 17).

When the MHC antigens were being studied on the tumour cells the lymphocyte gate was reversed to collect tumour cell data and exclude the lymphocytes (Fig. 18). This gate was also checked using the Leucogate preparation.

ANALYSIS

An irrelevant antibody control (goat anti-mouse IgG FITC and goat anti-mouse IgG PE) was used to set the analysis gates to exclude non-specific binding (Fig. 19).

The relative proportions of the phenotypic subsets were

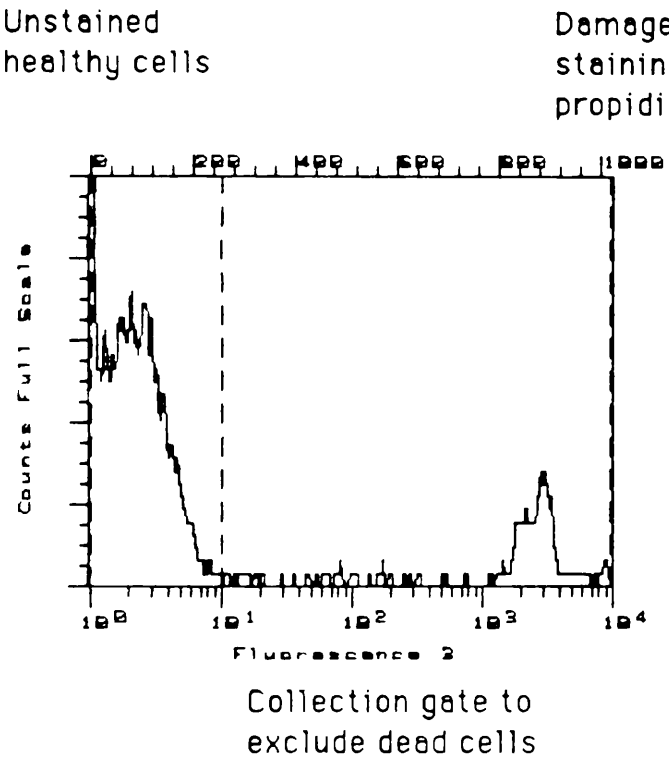


Fig. 16 Non-specific binding of antibodies to intracellular structures is avoided by the use of a collection gate which identifies dead cells by their PI staining and excludes them from data collection.

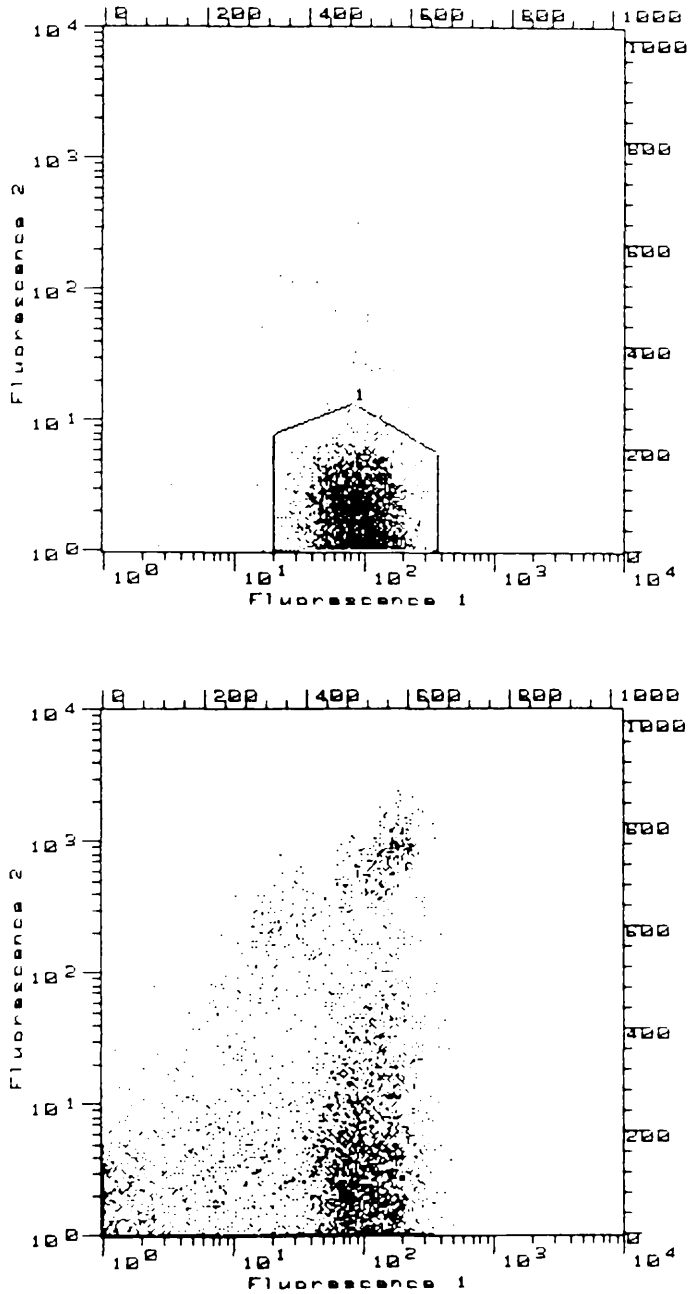


Fig. 17 The effectiveness of the lymphocyte gate is checked using Leucogate an antibody combination which differentially stains lymphocytes, monocytes and neutrophils. In the tumour samples this preparation is used to assess the degree of lymphocytic infiltration.

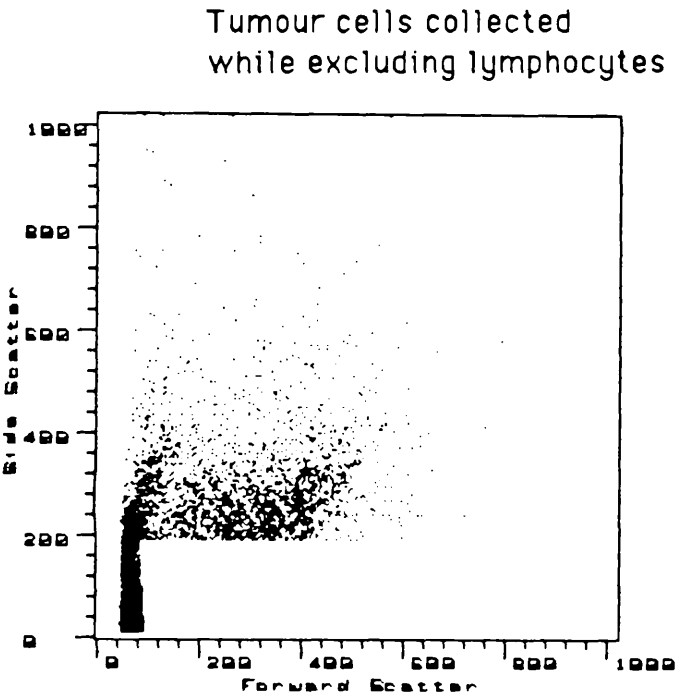
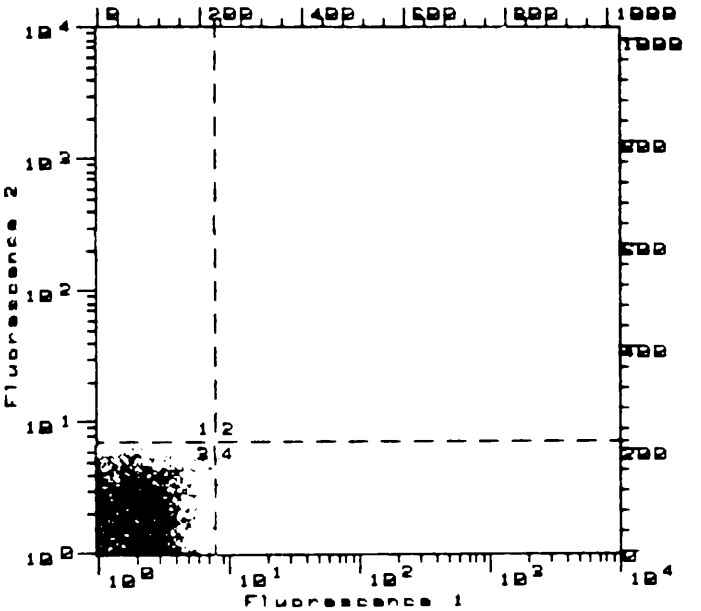


Fig.18 The lymphocyte gate is reversed to exclude lymphocytes from analysis of the markers borne by the tumour cells themselves.



Analysis markers set
on control sample.

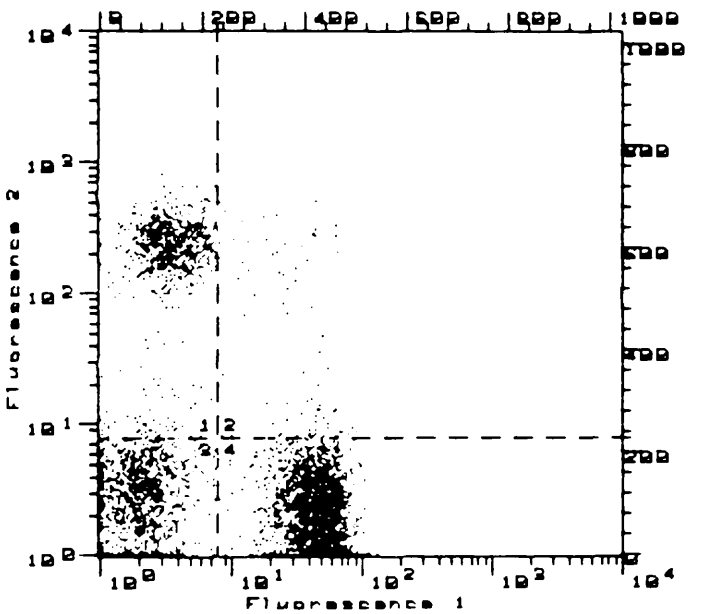
Fig. 19 To avoid non-specific binding giving false positive results, the quadrant analysis markers are set on the control sample to place 99.5% of the cells in the third quadrant at the origin.

analysed using four quadrant analysis (Fig. 20) while activation markers were measured by isolating the phenotype under study, using a PE conjugated antibody, and performing histogram analysis to identify the percentage of these cells carrying the FITC conjugated activation marker (Fig. 21). In both cases, the analysis gate for positive cells was based on the goat anti-mouse control for non-specific binding. Several patterns emerged, with some samples producing one histogram curve representative of a varied distribution of the activation marker (Fig. 21) while for other markers cells fell clearly into negative or positive populations (Fig. 22). In the case of HLA DR expression on B cells, while all B cells carried this marker, there appeared to be a cell population with a tenfold greater expression and this is what was measured (Fig. 23).

STATISTICAL ANALYSIS

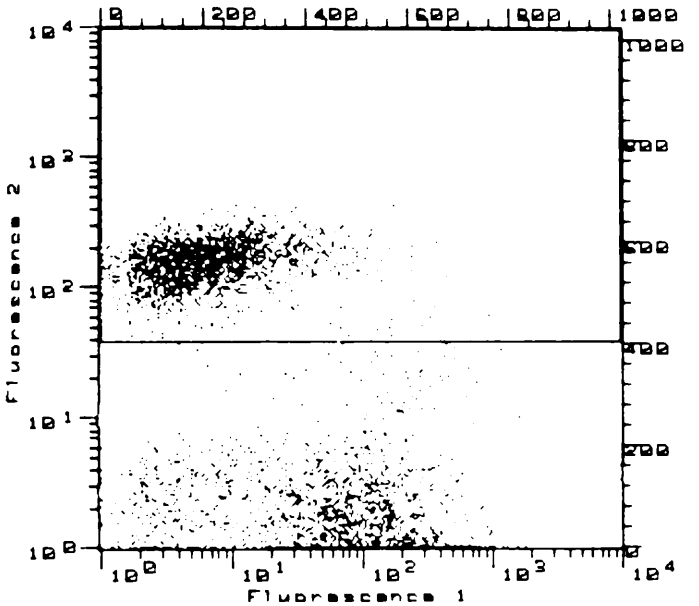
Due to the small number of subjects in the control group, the Student's t test was used to compare the peripheral blood and lymph node lymphocytes of these with those from the patients with breast cancer. This test was also used to compare the different lymphocyte

CD8+ T cells
suppressor/cytotoxic

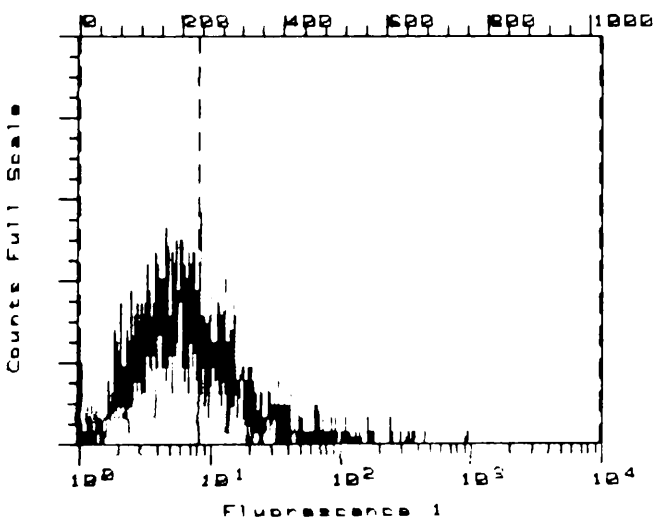


B cells CD4+ helper T cells

Fig. 20 Phenotypic proportions were analysed using quadrant statistics, seen here differentiating between CD4+ T cells, CD8+ T cells and unstained B cells.



Analysis gate used to isolate population of interest



Analysis marker based on non-specific FITC binding in control sample

Fig. 21 To analyse the activation marker expression of a phenotypic subgroup, the cells are isolated using an analysis gate and the number of cells bearing the activation marker is based on the control sample containing irrelevant antibody

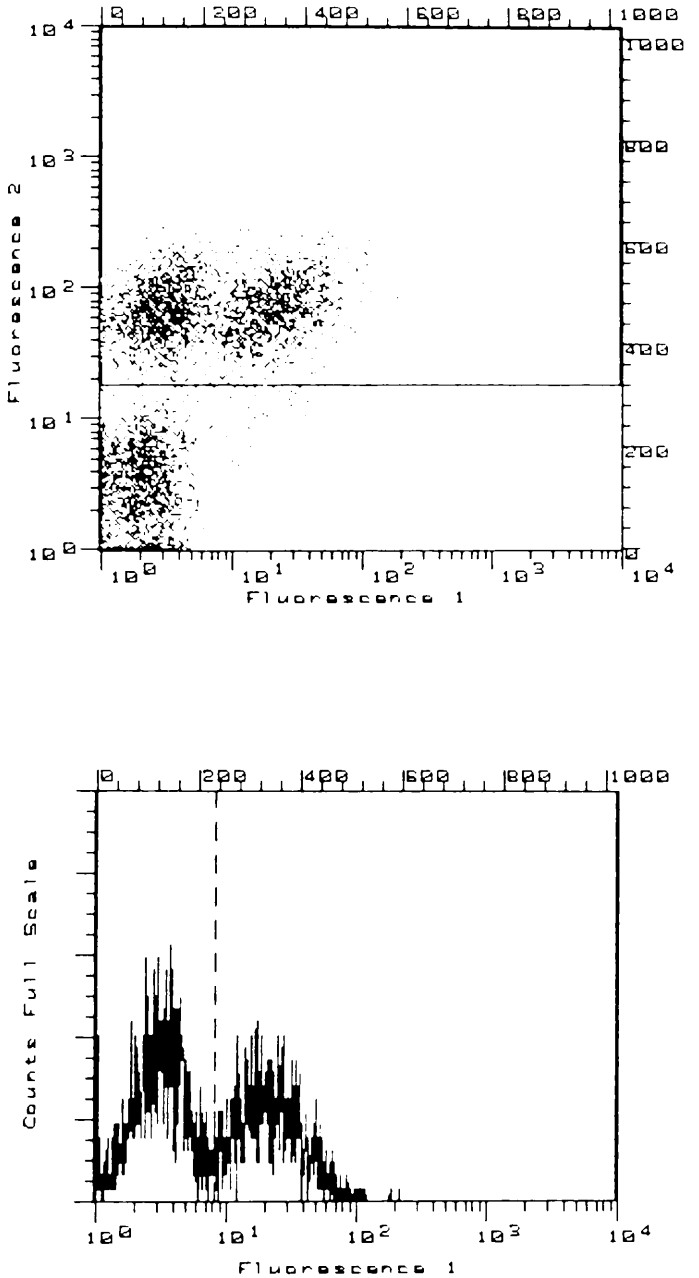


Fig. 22 Dot-plot and histogram appearance of IgG bearing B cells. As cells are either positive or negative for this surface immunoglobulin, they fall clearly into two distinct groups.

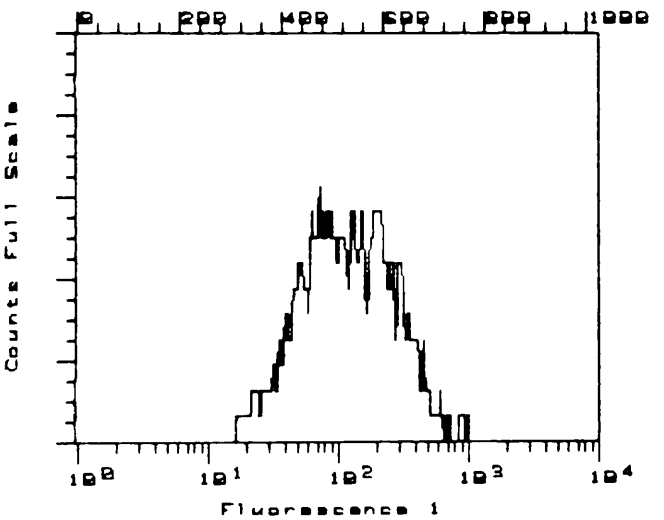


Fig. 23 Analysis of the HLA DR expression on B cells suggested the presence of two populations with one expressing higher levels of this marker. It was this population which was studied.

sources within the breast cancer patients. When the expression of surface markers was correlated with the expression of the MHC complexes or the known prognostic indicators of tumour stage, histological grade and oestrogen receptor status, the Spearman Rank correlation test was applied. This was also applied when correlation was sought between the surface marker expression on the PBLs and those from the regional nodes or primary tumour.

The data tables given in this study include the descriptive statistics of the mean and the standard error of the mean for the populations under comparison. Where comparison is by the Student's t test, the t value and significance (p value) are given. For the Spearman rank correlation, the r coefficient and significance are given and, where a significant correlation is found, the equation for the gradient of the line is also given. Statistical significance was taken at the 95% confidence level.

RESULTS

TUMOUR INFILTRATING LYMPHOCYTES

PHENOTYPIC MARKERS

Four of the tumours contained too few lymphocytes to allow the infiltrate even to be phenotyped leaving twenty seven with a scanty to strong infiltrate.
The lymphocytic infiltrate ranged from less than 1% of the cells harvested to 83%, with a mean of 10.5%.
Phenotypic analysis was performed using both the lymphocyte and live cell gates and phenotypic proportions are expressed as a percentage of the total lymphocyte population present within the tumour (Table 2). This analysis showed the tumour infiltrate to consist largely of T cells with only one tumour containing a significant number of B cells. This was also the tumour with the strongest lymphocytic infiltrate but no trend was seen among the other tumours.
When the T cells were further subdivided, the CD8+ suppressor/cytotoxic T cells were found to predominate with the CD4+/CD8+ ratio ranging from 0.2 to 2.1 and having an average of 0.8.

PARAMETER	DESCRIPTIVE STATISTICS	CORRELATION OF TIL SURFACE MARKERS WITH FEATURES OF PRIMARY TUMOUR. (n = 27)				
		STAGE	GRADE	E.R.	CLASS I	CLASSII
%TILs	10.5 ± 2.98	r=-0.094 p=0.616	r=0.102 p=0.592	r=-0.294 p=0.115	r=-0.06 p=0.748	r=0.012 p=0.947
%T cells	67.1 ± 2.73	r=-0.155 p=0.440	r=0.224 p=0.271	r=-0.10 p=0.626	r 0.416 p=0.031(c)	r=0.085 p=0.673
%B cells	11.5 ± 1.57	r=-0.019 p=0.923	r=0.051 p=0.806	r=-0.214 p=0.293	r=0.270 p=0.173	r=-0.113 p=0.574
%CD4 cells	30.3 ± 2.33	r=-0.210 p=0.292	r= 0.101 p=0.624	r=-0.202 p=0.322	r=0.396 p=0.041(d)	r=0.130 p=0.519
%CD8 cells	41.1 ± 2.91	r=-0.235 p=0.238	r=0.390 p=0.043(a)	r=-0.063 p=0.759	r=0.479 p=0.012(e)	r=0.183 p=0.360
CD4/CD8 ratio	0.86 ± 0.13	r=0.125 p=0.536	r=-0.499 p=0.01(b)	r=-0.125 p=0.542	r=-0.221 p=0.269	r=-0.061 p=0.762

Table 2 Correlation of the phenotypic proportions of the TILs with features of the primary lesion and stage of disease. There is some correlation of the CD8+ T cell population with tumour grade and both T cell subsets correlate with the expression of the Class I MHC antigen on the tumour cells.

CORRELATION GRADIENTS

(a) $y=-2.0909+7.5166x$ $R^2=0.191$

(b) $y=1.9051-0.4833x$ $R^2=0.246$

(c) $y=5.2731+0.3615x$ $R^2=0.198$

(d) $y=7.4941+0.4969x$ $R^2=0.089$

(e) $y=4.2154+0.6389x$ $R^2=0.217$

Only in seven tumours with a moderate or strong infiltrate were the helper T cells present in greater numbers (Fig. 24).

Although it did not achieve statistical significance, both phenotypes within the T cell infiltrate tended to diminish in patients with advancing disease stage.

With regard to oestrogen receptor (ER) status there was an inverse relationship, with oestrogen receptor negative tumours containing an average lymphocytic infiltrate which accounted for 15% of the cells while tumours which were oestrogen receptor positive contained 6% lymphocytes. This trend was seen in all T cell subsets but again did not achieve statistical significance.

When the infiltrating phenotypes were correlated with tumour grade there was found to be a positive correlation with the degree of infiltration by CD8+ suppressor/cytotoxic T cells ($p<0.05$) (Fig. 25). This alteration in the phenotypic constitution of the infiltrate, caused the CD4+/CD8+ ratio to decrease with increasing tumour grade ($p<0.01$) (Fig. 26).

No correlation was found between the proportion of tumour cells bearing the class II complex and the degree or phenotypic composition of the lymphocytic infiltrate but some correlation was found between the tumour cell expression of the class I MHC complex and the presence

Fig. 24

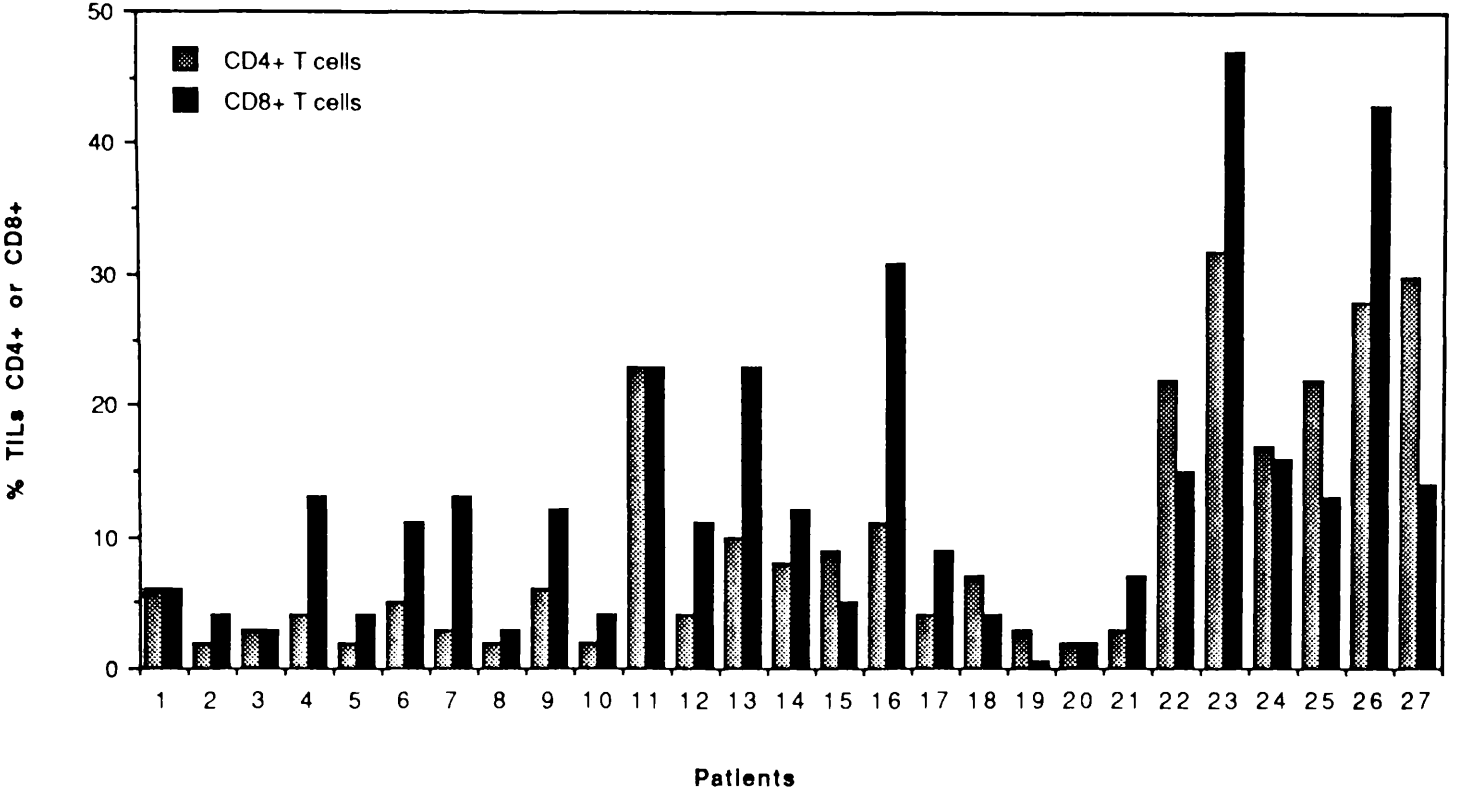

CD4+ T cells
CD8+ T cells
% TILs CD4+ or CD8+
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Patients

Fig. 25

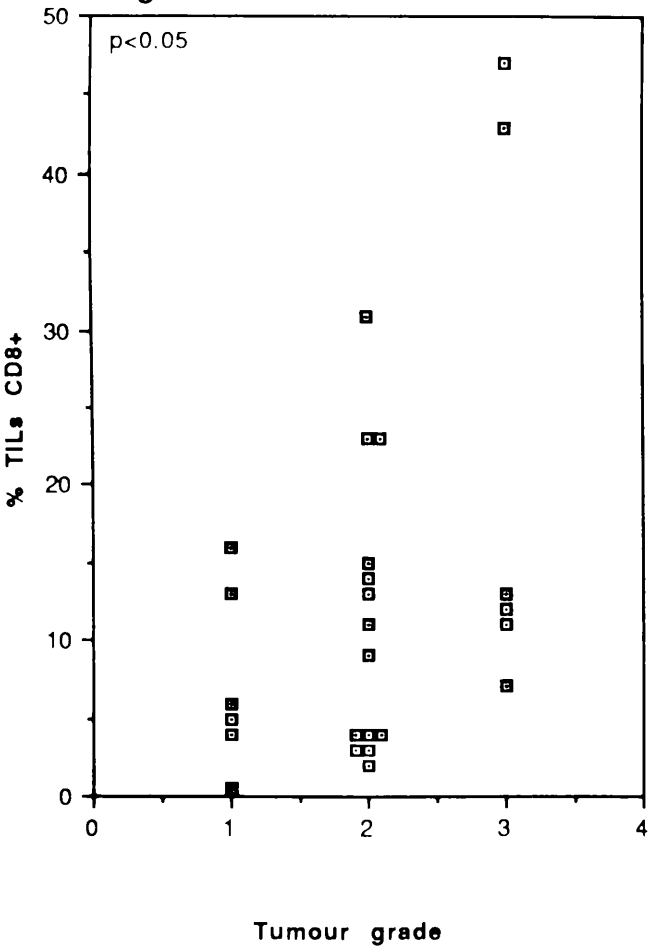
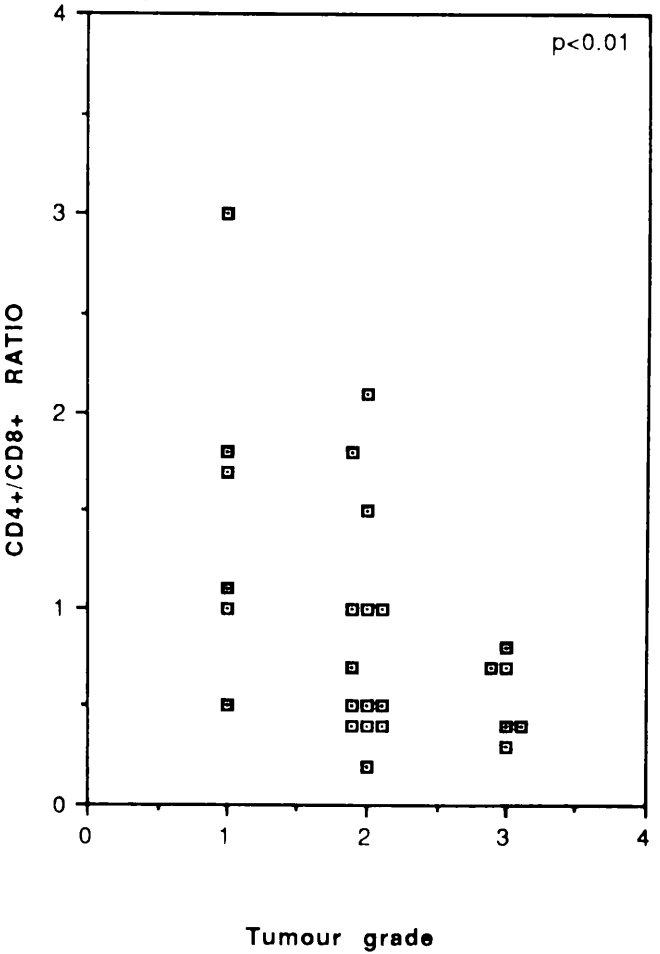

p<0.05
% TILs CD8+
0
10
20
30
40
50
0
1
2
3
4
Tumour grade

Fig. 26


p<0.01
CD4+/CD8+ RATIO
0
1
2
3
4
0
1
2
3
4
Tumour grade

of a lymphocytic infiltrate ($p=0.05$).

When this tumour marker was correlated with the T cell subset proportions it was found that, while it did correlate with the number of CD4+ helper T cells infiltrating the tumour ($p<0.05$) (Fig. 27), there was a stronger correlation with the size of the CD8+ suppressor/cytotoxic T cell population ($p<0.01$) (Fig. 28).

ACTIVATION MARKERS

HLA DR

This marker was found to be was present on 49% of the CD4+ helper T cells and 57% of the CD8+ suppressor/cytotoxic T cells infiltrating the tumours. No relationship was found with tumour stage or ER status but a strong correlation was found with tumour grade (Table 3). This relationship between grade and the number of cells expressing HLA DR was seen in both subsets although it was stronger among CD8+ suppressor/cytotoxic T cells ($p=0.003$) (Fig. 29a) than among the CD4+ helper T cells ($p=0.03$) (Fig. 29b). This marker was also found to correlate strongly with the tumour expression of both the class I and class II MHC complexes. The number of CD4+ cells bearing HLA DR

Fig. 27

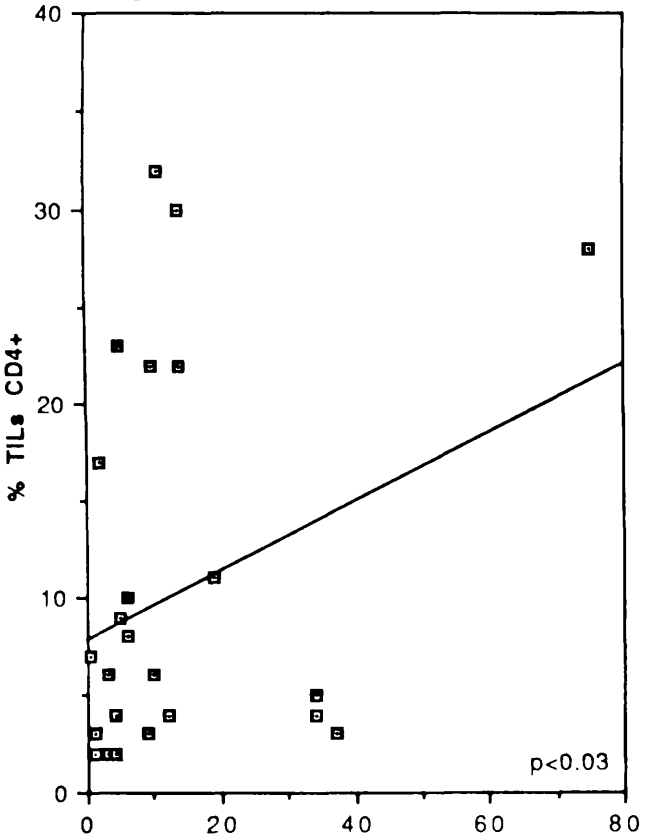
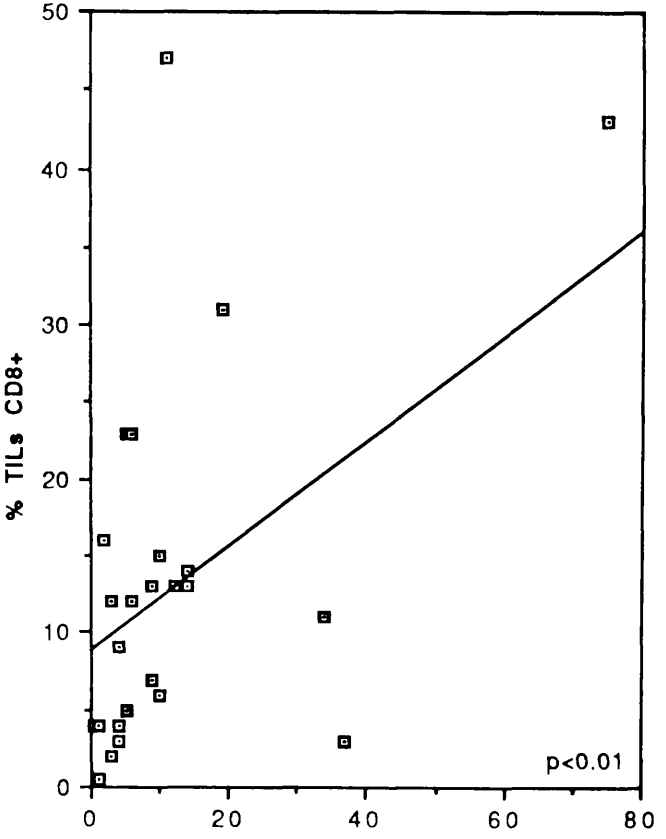

40
30
20
10
0
% TILs CD4+
0
20
40
60
80
p<0.03
Tumour Class I MHC expression

Fig. 28


50
40
30
20
10
0
% TILs CD8+
0
20
40
60
80
p<0.01
Tumour Class I MHC expression

PARAMETER	DESCRIPTIVE STATISTICS	CORRELATION OF TIL SURFACE MARKERS WITH FEATURES OF PRIMARY TUMOUR. (n = 22)				
		STAGE	GRADE	E.R.	CLASS I	CLASSII
%CD8+DR+	56.9 ± 3.59	r=-0.113 p=0.618	r=0.611 p=0.003(a)	r=-0.064 p=0.782	r=0.693 p<0.0001(c)	r=0.823 p<0.0001(f)
%CD8+Tac+	13.7 ± 1.36	r=0.154 p=0.495	r=-0.047 p=0.838	r=0.226 p=0.324	r=-0.207 p=0.356	r=0.034 p=0.881
%CD8+TrfR	38.1 ± 2.15	r=0.150 p=0.553	r=0.134 p=0.608	r=0.041 p=0.877	r=0.178 p=0.479	r=0.294 p=0.236
%CD4+DR+	48.6 ± 3.80	r=-0.244 p=0.275	r=0.472 p=0.031(b)	r=-0.009 p=0.969	r=0.548 p=0.008(d)	r=0.518 p=0.014(g)
%CD4+Tac+	27.6 ± 1.31	r=-0.031 p=0.889	r=0.091 p=0.694	r=-0.174 p=0.452	r=0.415 p=0.049(e)	r=0.487 p=0.022(h)
%CD4+TrfR	48.6 ± 4.23	r=0.202 p=0.423	r=-0.098 p=0.709	r=0.072 p=0.784	r=0.064 p=0.801	r=0.218 p=0.385

Table 3 Correlation of the activation marker expression on the TILs and the features of the primary lesion. There is strong correlation between the T cell subset expression of HLA DR and both tumour grade and the tumour cell expression of the Class I and II MHC antigens.

CORRELATION GRADIENTS

(a) $y=28.617+14.489x$ $R^2=0.364$
(b) $y=22.830+13.106x$ $R^2=0.251$
(c) $y=-23.845+0.637x$ $R^2=0.413$
(d) $y=-11.734+0.497x$ $R^2=0.281$
(e) $y=25.485+0.1657x$ $R^2=0.203$
(f) $y=-27.924+0.735x$ $R^2=0.523$
(g) $y=-3.1072+0.351x$ $R^2=0.133$
(h) $y=25.00+0.1827x$ $R^2=0.259$

Fig. 29a

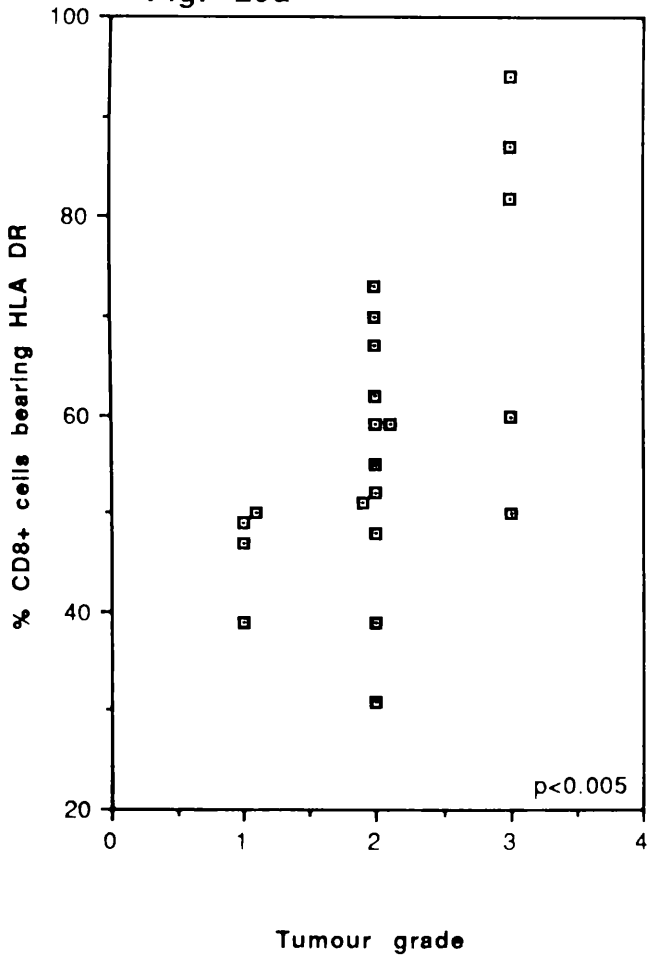
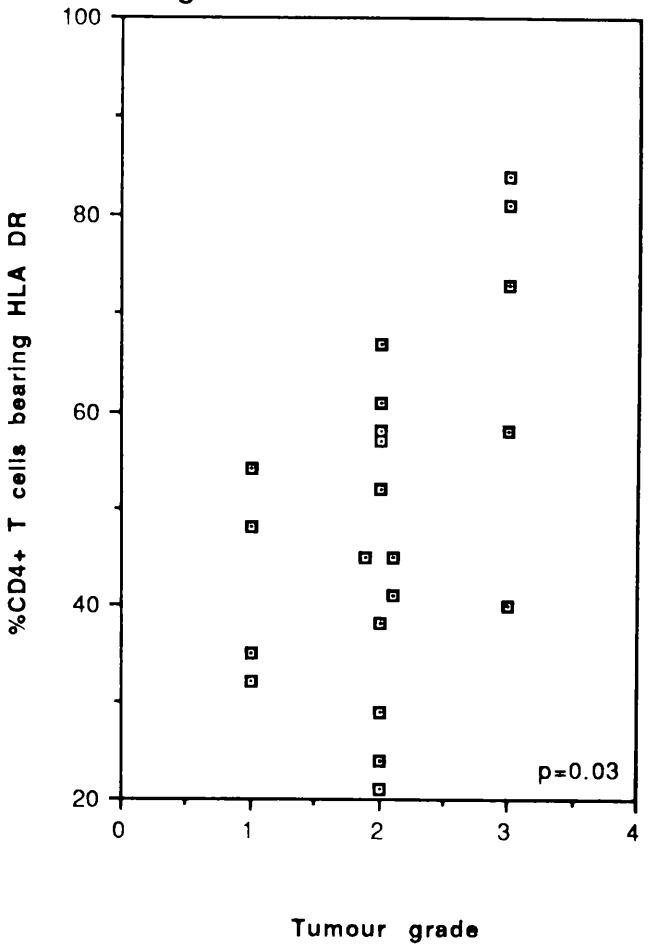


Fig. 29b



correlated with the proportion of tumour cells bearing the class I ($p<0.01$) (Fig. 30a) and class II antigens ($p<0.02$) (Fig. 30b). The correlation of HLA DR expression on CD8+ T cells with the presence of tumour histocompatibility antigens was even stronger with $p<0.0001$ for both class I and class II complexes (Figs. 31a & 31b).

HLA DR tended to be carried by a greater proportion of suppressor/cytotoxic CD8+ T cells in tumours with a strong CD8+ T cell component to their infiltrate ($p<0.01$) (Fig. 32) and was present on up to 90% of the tumour infiltrating CD8+ T cells in some patients.

Interleukin 2 receptor

The Tac antigen was present on 14% of the CD8+ cytotoxic T cells in the infiltrate but was consistently found on a higher proportion of the CD4+ helper T cells (mean 28%) ($p<0.0001$) (Fig. 33).

There was no relationship detected between this marker and tumour stage, grade or ER status but its presence on the CD4+ helper T cells correlated with the proportion of tumour cells bearing the class I ($p<0.05$) and class II ($p<0.03$) MHC complexes (Figs. 34a & 34b).

This marker was also present on a greater proportion of CD4+ helper T cells as the number of these cells within the tumour increased ($p<0.05$) (Fig. 35).

Fig. 30a

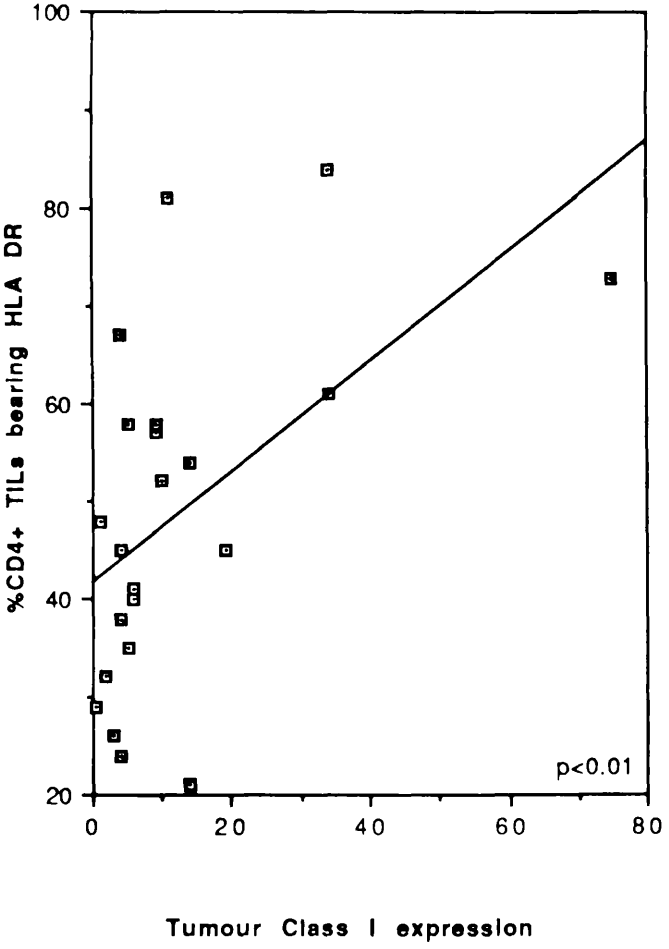


Fig. 30b

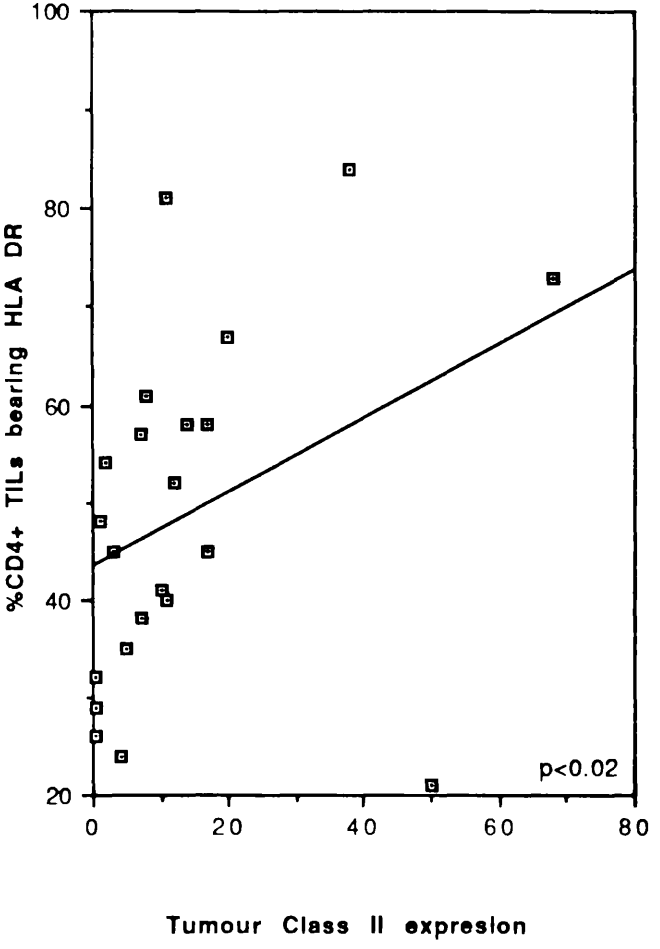


Fig. 31a

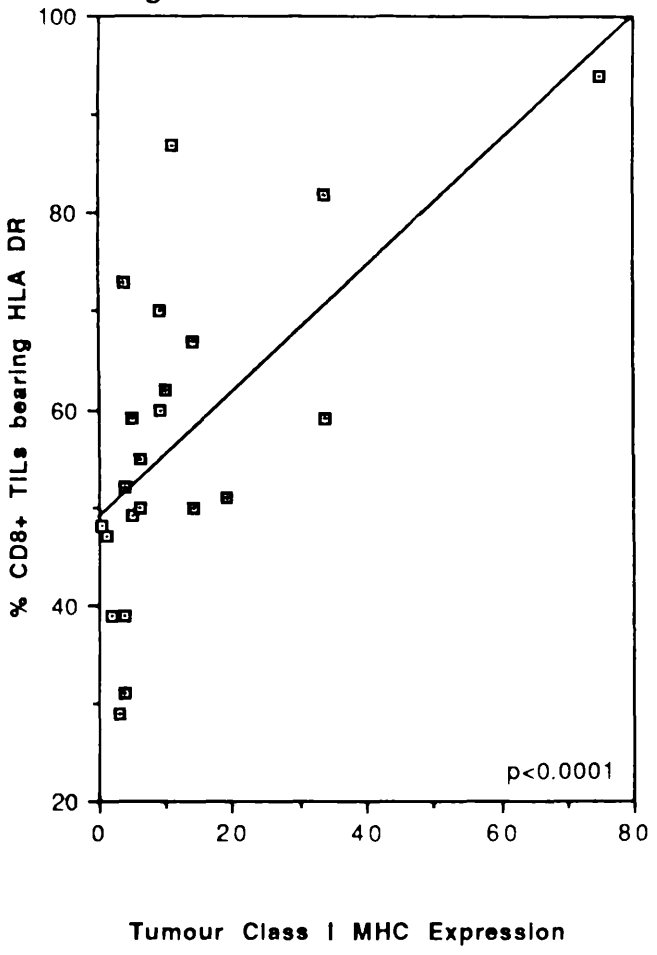


Fig. 31b

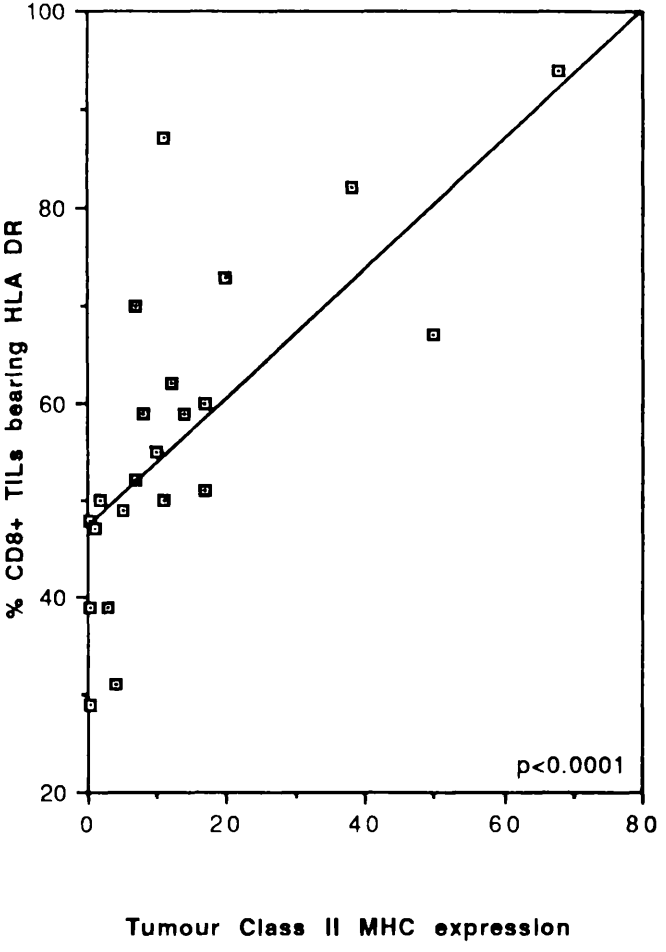


Fig. 32

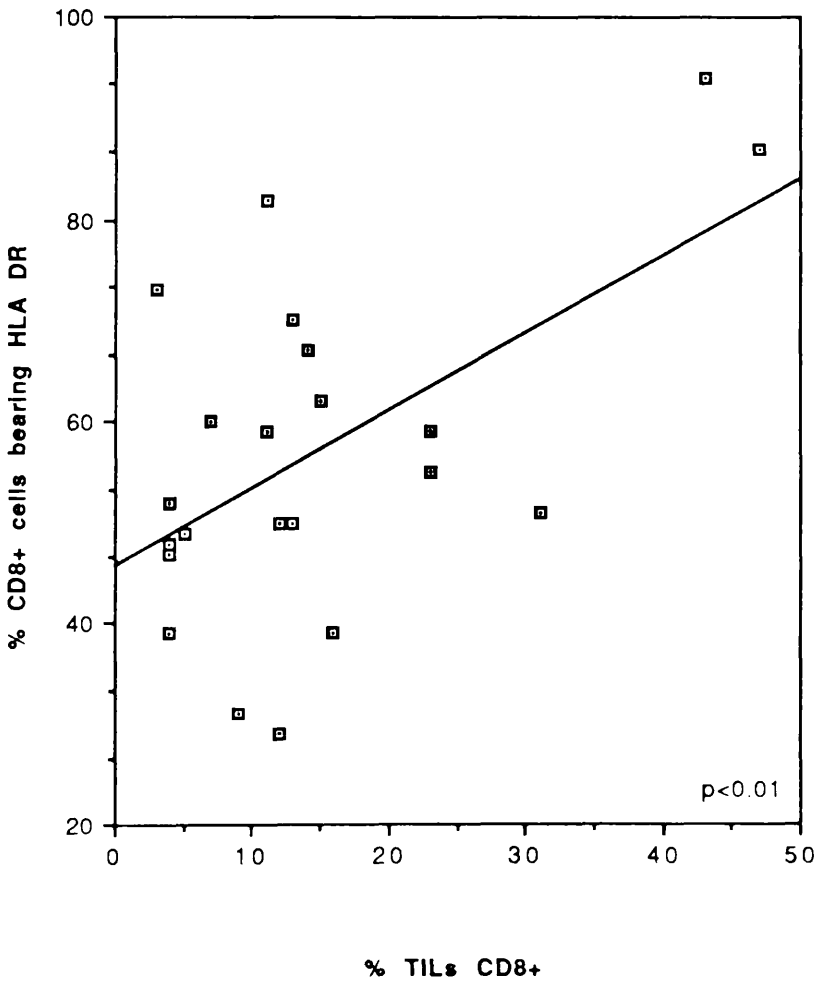

100
80
60
40
20
0
10
20
30
40
50
% CD8+ cells bearing HLA DR
% TILs CD8+
p<0.01

Fig. 33

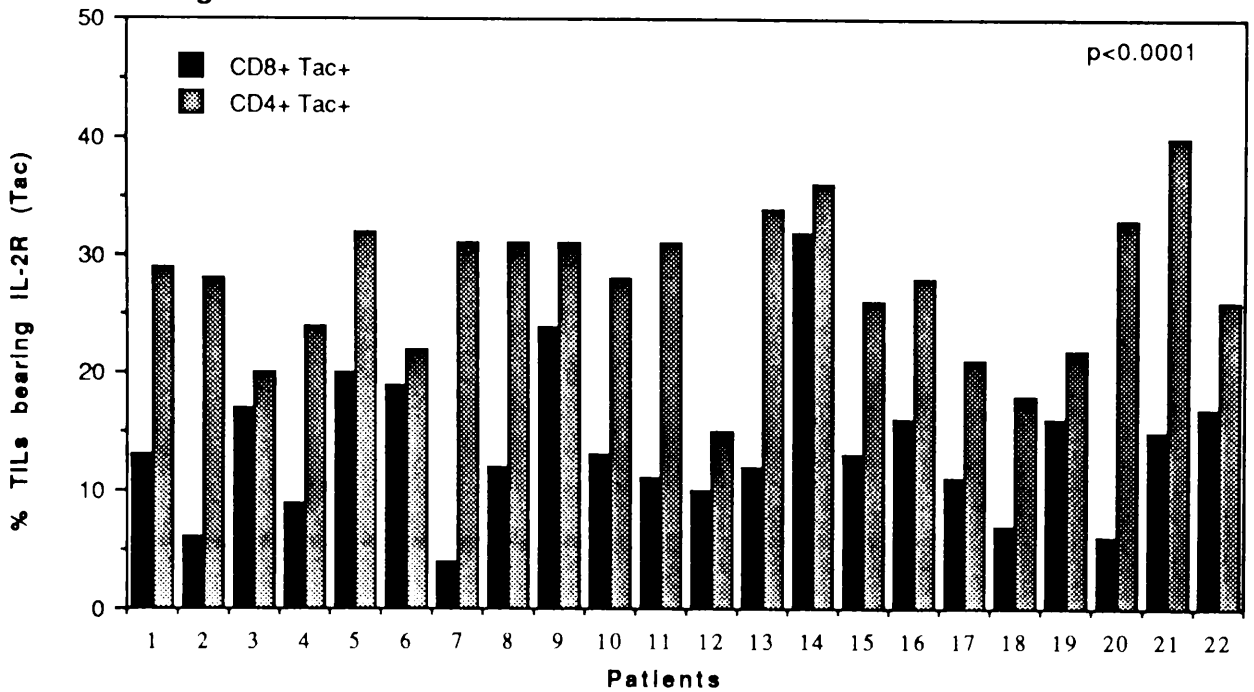

CD8+ Tac+
CD4+ Tac+
p<0.0001
% TILs bearing IL-2R (Tac)
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Patients

Fig. 34a

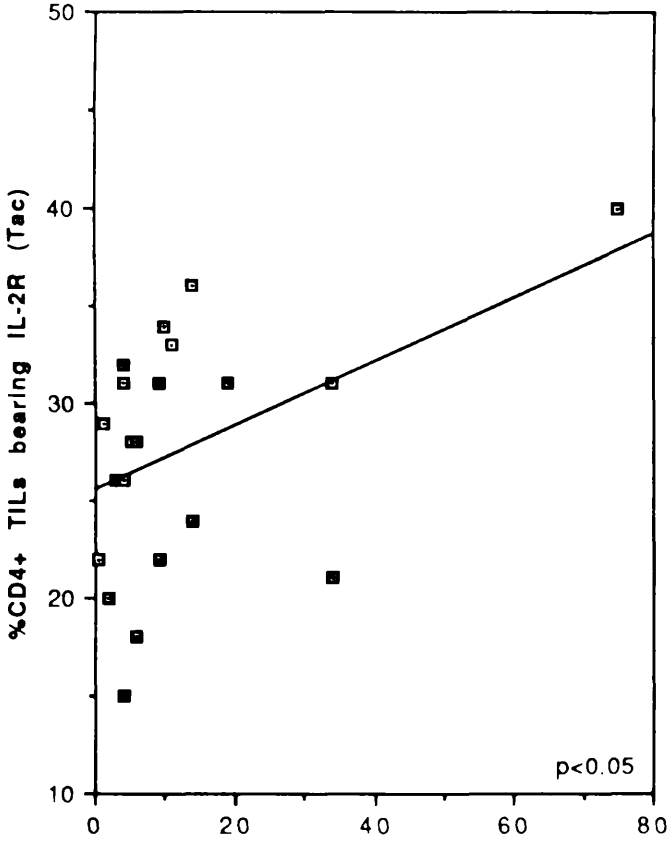


Fig. 34b

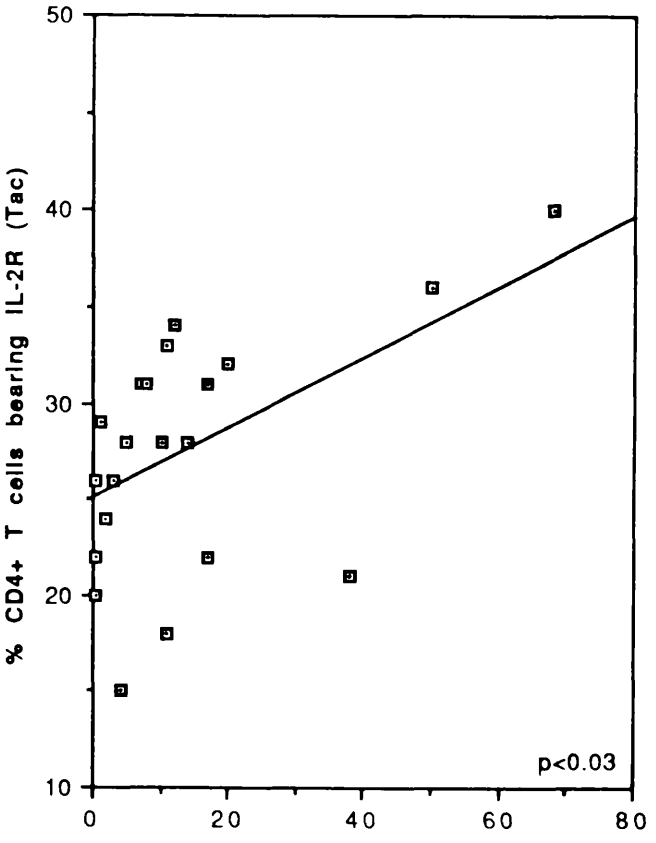
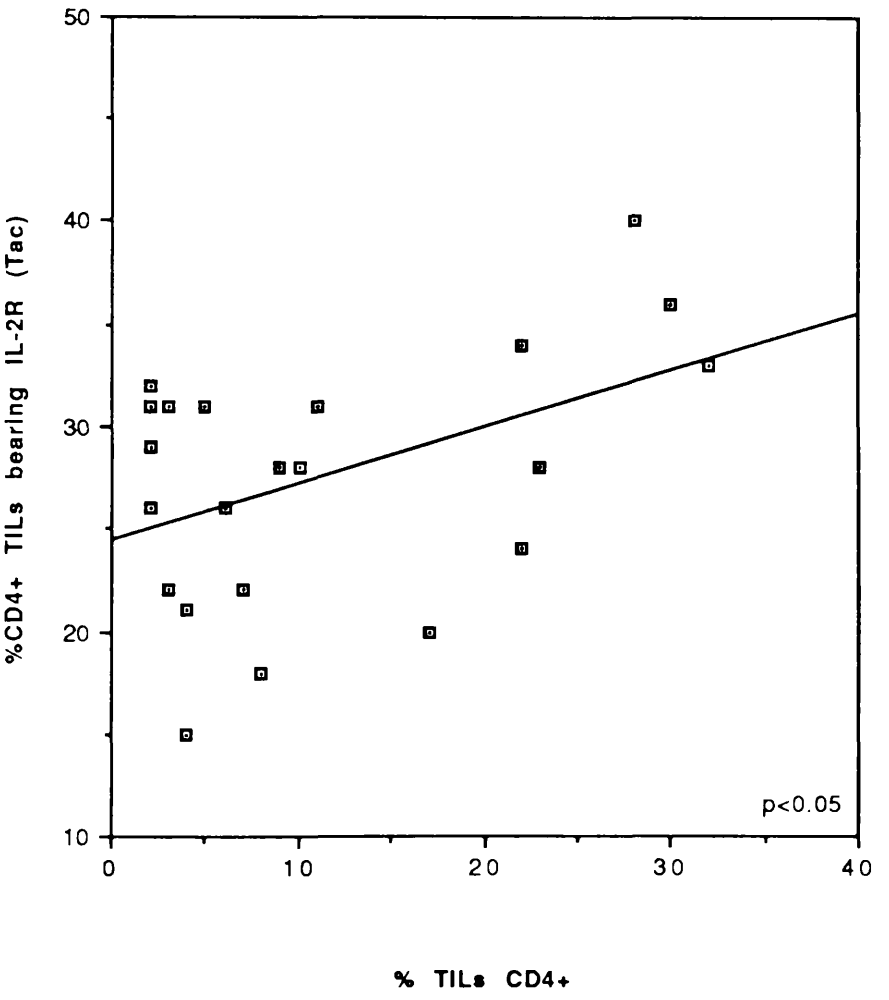


Fig. 35


50
40
30
20
10
0
10
20
30
40
%CD4+ TILs bearing IL-2R (Tac)
% TILs CD4+
p<0.05

Transferrin receptor

The transferrin receptor was present on the surface of 39% of the CD8+ suppressor/cytotoxic T cells and 48% of the CD4+ helper T cells within the tumour. This marker did not relate to the histological grade, stage of disease or ER status, nor to the proportion of tumour cells bearing the class I or class II MHC complexes.

Immunoglobulin G

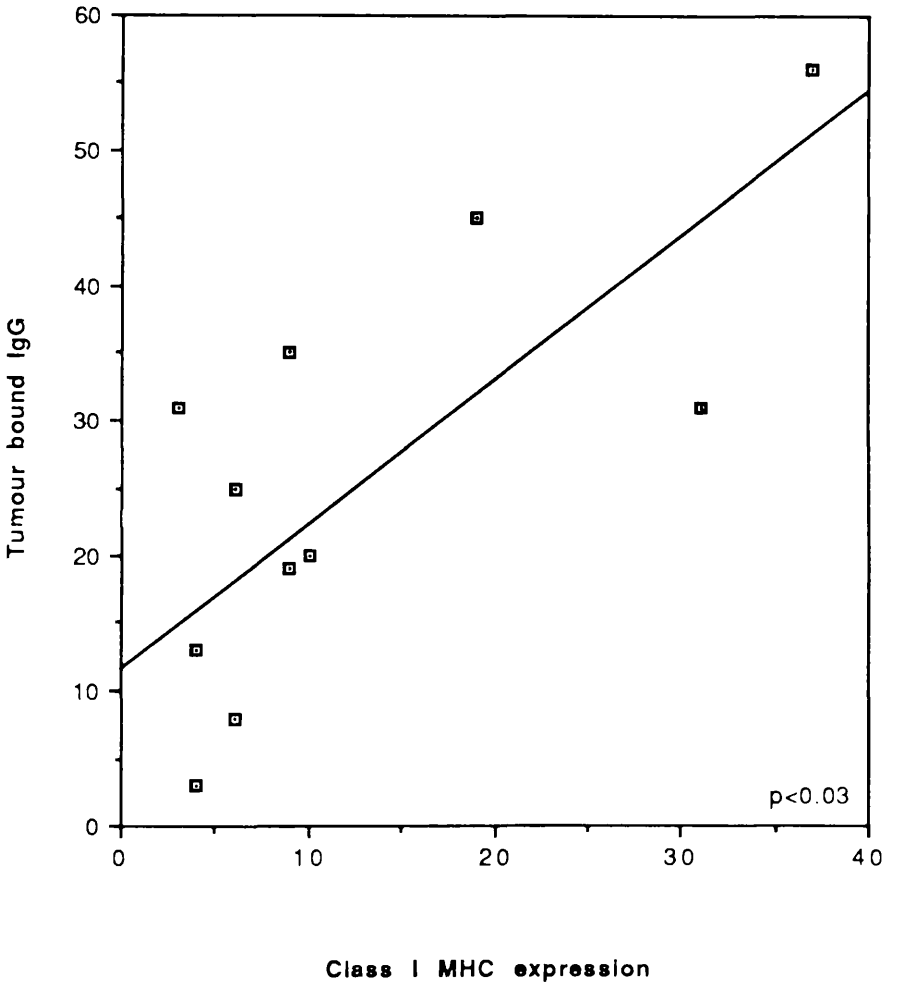
This was found on an average of 28% of the tumour cells, with a range from 3% to 81%. The presence of IgG on the tumour cells was not related to tumour stage, grade or ER expression, nor to the proportion of tumour cells bearing the class II MHC complex but did correlate with the number of tumour cells carrying the class I antigen ($p<0.03$) (Fig. 36).

LYMPH NODE LYMPHOCYTES

PHENOTYPIC MARKERS

The axillary lymph nodes from the breast cancer patients were found to be large and engorged with an average cell yield of $4x10^7$ from each half while normal nodes were small and pale with cell counts of less than $1x10^7$.

Fig. 36


60
50
40
30
20
10
0
Tumour bound IgG
0
10
20
30
40
p<0.03
Class I MHC expression

From the results given in Table 4 it can be seen that, when lymph node lymphocytes (LNLs) from breast cancer patients were compared with those from normal controls, there was some alteration in the phenotypic proportions. In this study no significant differences were found in the proportion of T or B lymphocytes between the lymph nodes of patients with breast cancer and those from normal controls.

However, when the T cell population was further subdivided, there was found to be a great increase in the CD4+ helper T cell population in the nodes of cancer patients, with a mean of 49% as opposed to 33% in the normal controls ($p=0.003$) (Fig. 37) and a slight decrease in the proportion of CD8+ suppressor/cytotoxic T cells with a mean of 12% in the patients and 17% in the controls ($p=0.01$).

While the CD4+/CD8+ ratios for the normal nodes were within a tight range with a mean of 2, those of the patients' nodes ranged widely from 1.22 to 16.25 and had an average of 4.86 ($p=0.02$) (Fig. 38). This broad range of CD4+/CD8+ ratios in the patient group appeared to be a function of the variation in the size of the CD4+ helper T cell population from 22% to 69% with less variation in the size of the CD8+ suppressor/cytotoxic population.

There appeared to be no relationship between the

PARAMETER	PATIENT LYMPH NODE n = 40	CONTROL LYMPH NODE n = 7	STUDENT'S *t* TEST
% T cells	59.2 ± 2.06	50.7 ± 5.20	t = 1.584 p = 0.120
% B cells	35.0 ± 2.01	39.6 ± 5.65	t = -0.866 p = 0.391
% CD4+ T cells	49.0 ± 1.95	33.1 ± 4.05	t = 3.200 p = 0.003
% CD8+ T cells	12.2 ± 0.65	16.9 ± 1.98	t = -2.671 p = 0.01
CD4+/CD8+ ratio	4.9 ± 0.49	2.0 ± 0.15	t = 2.394 p = 0.021

Table 4 Comparing the phenotypic proportions of lymph nodes from patients with breast cancer and normal subjects.

Fig. 37

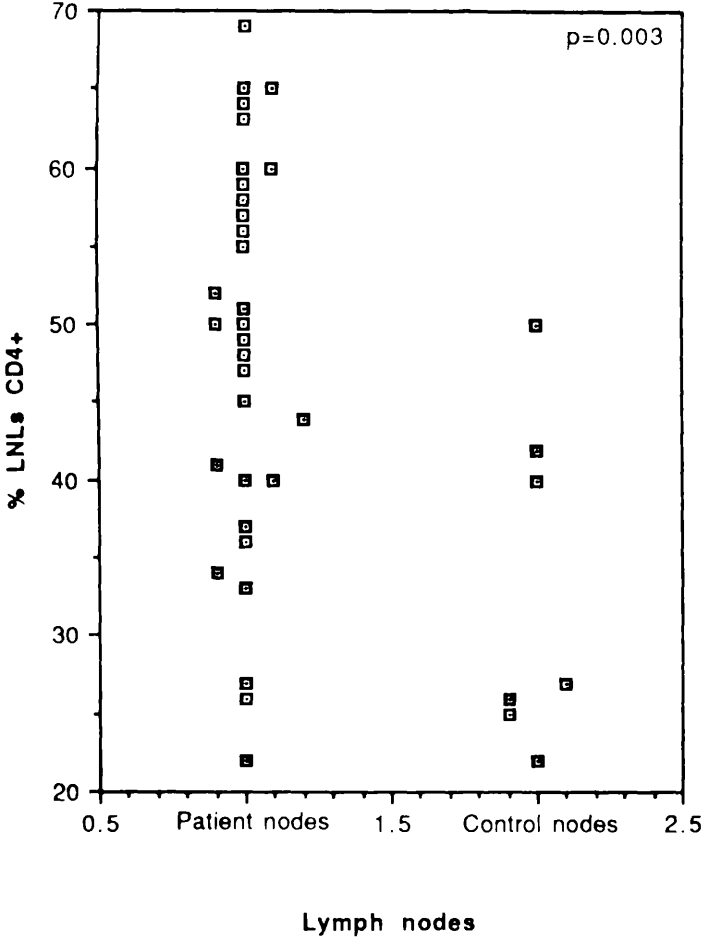
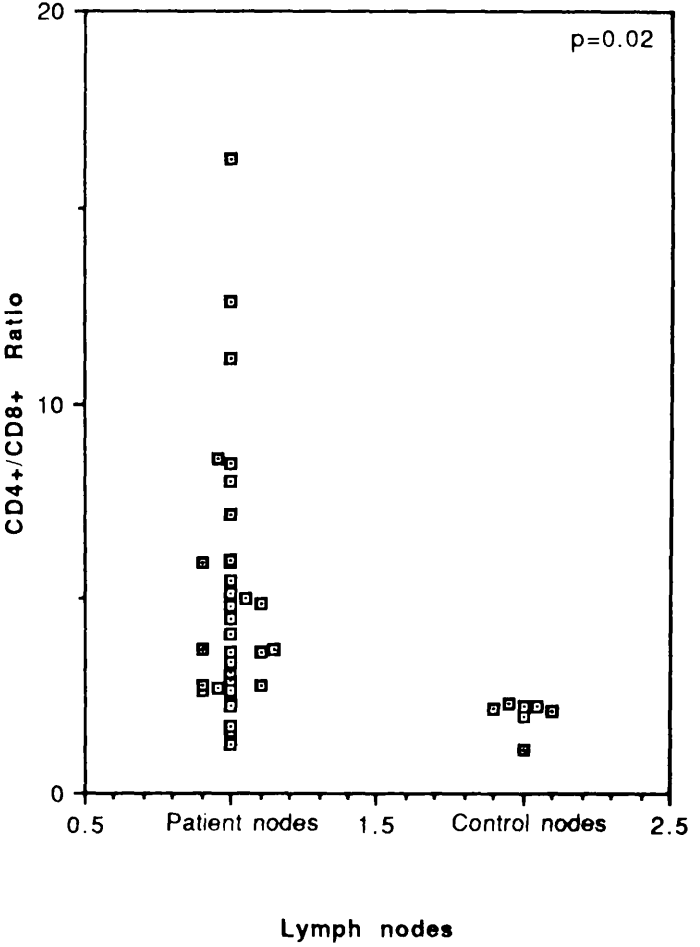

p=0.003
% LNLs CD4+
70
60
50
40
30
20
0.5
Patient nodes
1.5
Control nodes
2.5
Lymph nodes

Fig. 38


p=0.02
CD4+/CD8+ Ratio
20
10
0
0.5
Patient nodes
1.5
Control nodes
2.5
Lymph nodes

phenotypic proportions and the stage of the disease.

ACTIVATION MARKERS

HLA DR

As can be seen in Table 5, this surface marker was found to be on many more LNLs from the lymph nodes of patients with breast cancer than from the control nodes. This increase was largely among the T lymphocytes with 49% of the CD8+ T cells from patients and only 18% of those from control nodes bearing this marker ($p<0.0001$) while 38% of the CD4+ T cells from the patients bore this antigen compared with 12% from the nodes of normal controls ($p<0.0001$) (Fig. 39). This marker tended to be present on more CD8+ than CD4+ T cells ($p<0.001$). Virtually all B lymphocytes carry the HLA DR antigen and there appeared to be no difference in the number of B cells with high HLA DR expression between the two groups.

The expression of HLA DR on the CD8+ T cell population correlated with tumour stage, increasing in patients with nodal metastases ($p=0.02$) (Table 6; Fig. 40).

PARAMETER	PATIENT LYMPH NODE n = 40	CONTROL LYMPH NODE n = 7	STUDENT'S *t* TEST
CD8+ T cells bearing HLA DR	48.6 ± 2.09	18.3 ± 2.25	t = 5.910 p < 0.0001
CD8+ T cells bearing Tac	16.0 ± 1.07	8.1 ± 1.75	t = 2.921 p = 0.005
CD8+ T cells bearing TrfR	33.7 ± 2.01	31.3 ± 13.59	t = 0.332 p = 0.742
CD4+ T cells bearing HLA DR	37.5 ± 1.59	12.1 ± 2.61	t = 6.367 p < 0.0001
CD4+ T cells bearing Tac	22.5 ± 1.07	14.4 ± 2.82	t = 2.883 p = 0.006
CD4+ T cells bearing TrfR	29.1 ± 2.16	22.0 ± 6.76	t = 0.989 p = 0.328
B cells with high HLA DR	26.7 ± 2.09	19.1 ± 5.04	t = 1.383 p = 0.173
B cells bearing Tac	16.6 ± 1.58	15.3 ± 4.09	t = 0.330 p = 0.743
B cells bearing TrfR	43.6 ± 1.86	33.3 ± 6.18	t = 1.670 p = 0.102
B cells bearing surface IgG	30.6 ± 2.51	17.1 ± 2.51	t = 2.195 p = 0.033

Table 5 Comparison of the activation marker expression on lymphocytes from the lymph nodes of patients with breast cancer and normal subjects.

PARAMETER LNLs (n = 40)	CORRELATION WITH TUMOUR STAGE (Spearman Rank correlation)		CORRELATION GRADIENT
% T cells	r = -0.204	p = 0.363	NS
% B cells	r = 0.215	p = 0.336	NS
% CD4+ T cells	r = -0.072	p = 0.751	NS
% CD8+ T cells	r = -0.274	p = 0.217	NS
CD4+/CD8+	r = 0.085	p = 0.706	NS
%CD8+ HLA DR+	r = 0.486	p = 0.022	y=38.426+6.7794x R^2 = 0.210
%CD8+ Tac+	r = 0.108	p = 0.634	NS
%CD8+ TrfR+	r = 0.245	p = 0.272	NS
%CD4+ HLA DR+	r = 0.168	p = 0.456	NS
%CD4+ Tac+	r = 0.257	p = 0.247	NS
%CD4+ TrfR+	r = 0.325	p = 0.140	NS
%B high HLA DR	r = 0.148	p = 0.512	NS
%B Tac+	r = -0.330	p = 0.144	NS
%B TrfR+	r = 0.071	p = 0.754	NS
%B IgG+	r = -0.096	p = 0.672	NS

Table 6 Correlation of the surface markers borne by lymph node lymphocytes and tumour stage. The expression of HLA DR on the lymph node CD8+ T cells increases with the development of nodal metastases.

Fig. 39

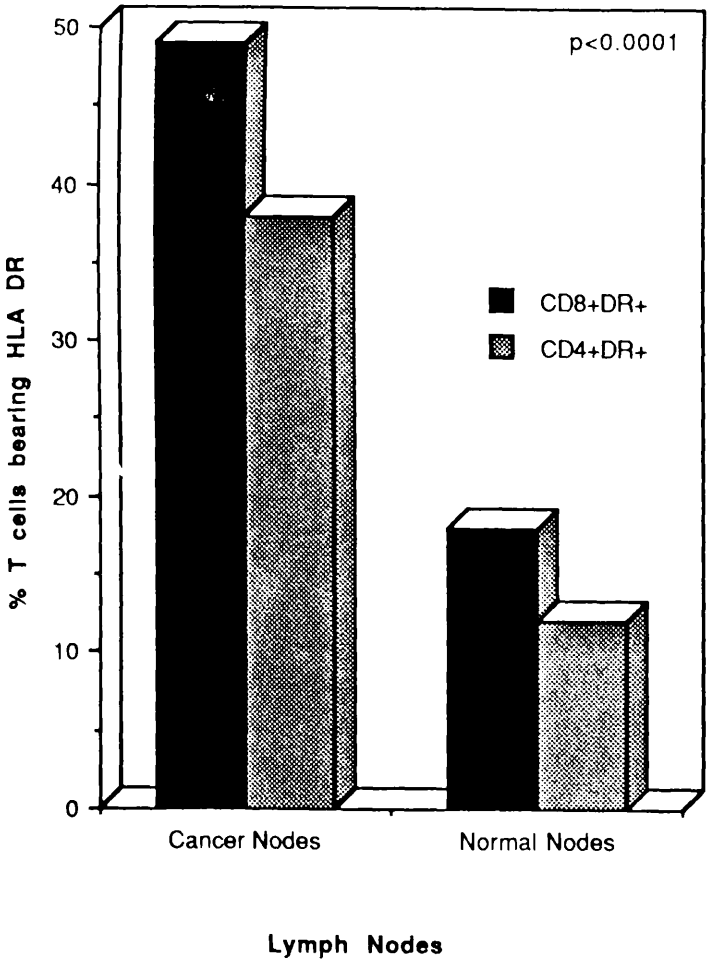
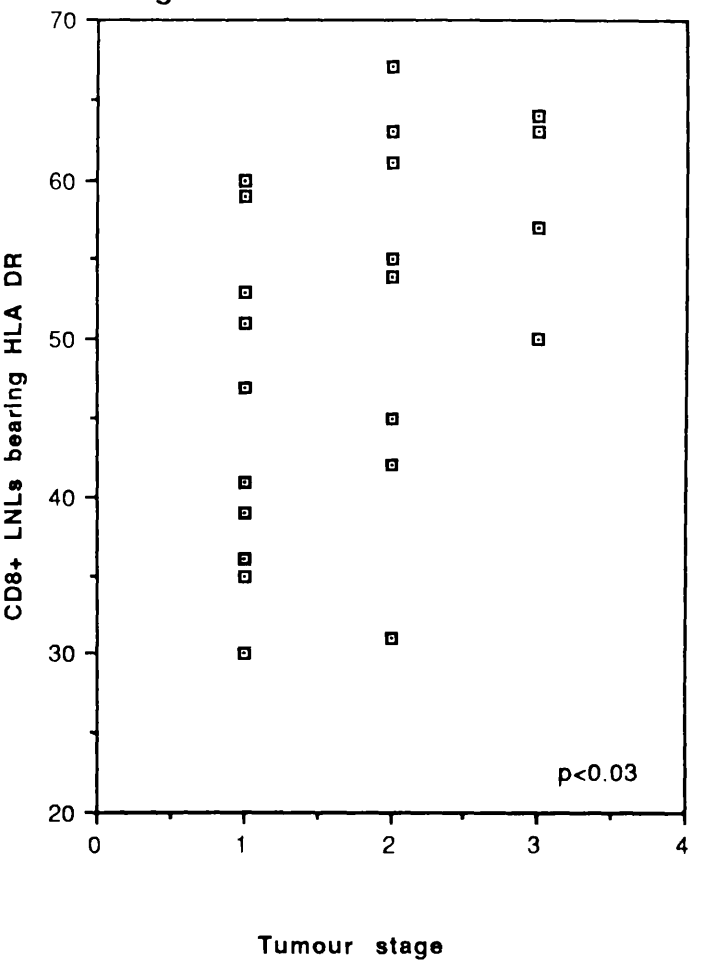

p<0.0001
CD8+DR+
CD4+DR+
% T cells bearing HLA DR
50
40
30
20
10
0
Cancer Nodes
Normal Nodes
Lymph Nodes

Fig. 40


CD8+ LNLs bearing HLA DR
70
60
50
40
30
20
p<0.03
0
1
2
3
4
Tumour stage

Interleukin 2 receptor

This marker was also found on many more T lymphocytes from the lymph nodes of breast cancer patients than from normal subjects, while there was no difference in the number of B lymphocytes carrying this marker in the two groups.

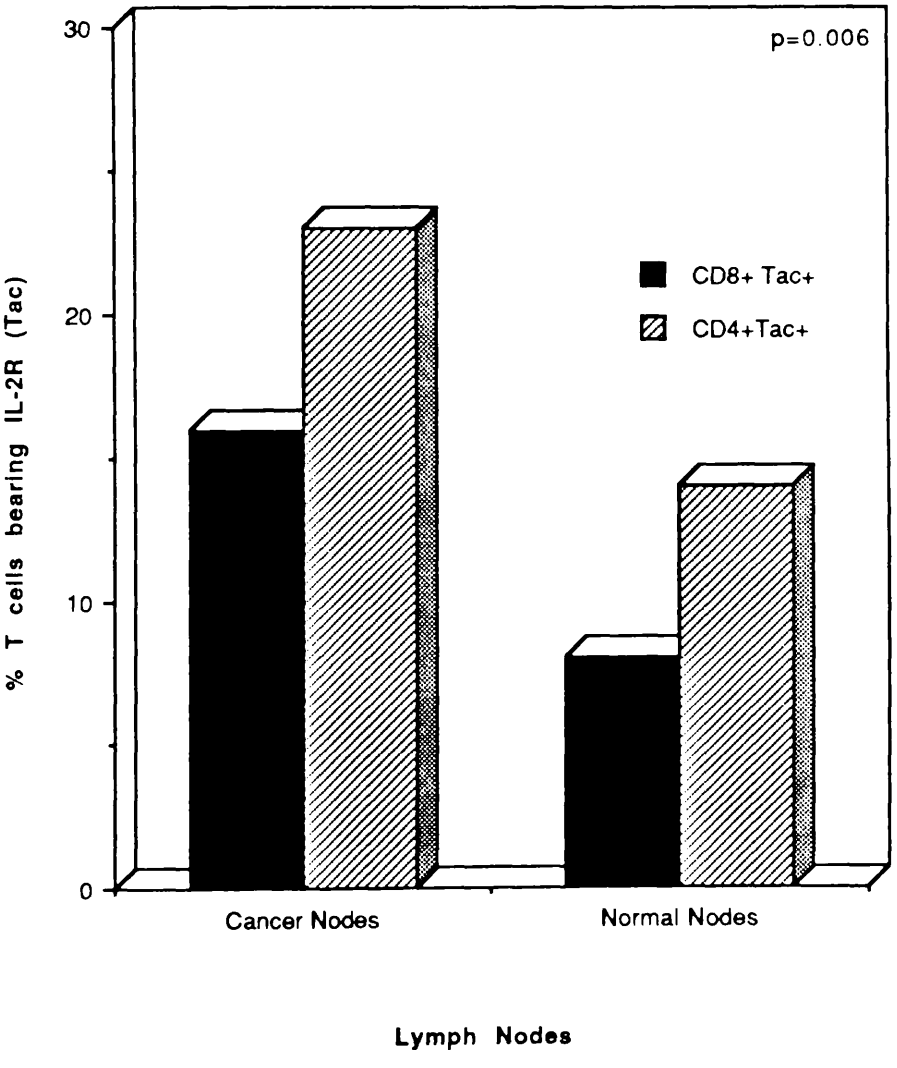
When the T cell subsets were studied, it was seen that this marker increased from being carried on 8% of CD8+ T cells in the control subjects to 16% of CD8+ T cells in the patient group ($p=0.005$) and from 14% of control CD4+ T cells to 23% of CD4+ T cells in the nodes of breast cancer patients ($p=0.006$) (Fig. 41). It was again noted that more CD4+ than CD8+ T cells carried this marker.

The presence of this receptor on the lymph node T cells of breast cancer patients did not appear to be affected by spread of the tumour to the regional nodes.

Transferrin receptor

As can be seen in Table 5, more lymphocytes, of all phenotypes, bore this marker in the nodes of patients with breast cancer than in the normal controls but this did not achieve statistical significance. There was no relationship with tumour stage.

Fig. 41


p=0.006
30
20
10
0
% T cells bearing IL-2R (Tac)
CD8+ Tac+
CD4+Tac+
Cancer Nodes
Normal Nodes
Lymph Nodes

Immunoglobulin G

A marked increase in the number of B lymphocytes carrying surface IgG was found in the breast cancer patients compared with the controls. The number of IgG committed B cells in the lymph nodes of normal subjects varied within a fairly narrow range from 8% to 24% with a mean of 17% while in the cancer patients the mean was higher at 31% with a much wider range from 3% to 70% ($p=0.03$) (Fig. 42). The number of B lymphocytes present in the lymph nodes correlated with the number bearing surface IgG ($p=0.04$) (Fig. 43). No correlation was found between the number of IgG committed B lymphocytes and tumour stage.

PERIPHERAL BLOOD

PHENOTYPIC MARKERS

Similar lymphocyte yields were obtained from the blood samples of breast cancer and normal patients.
T lymphocytes were found to be the major cell type making up 59% of the cells in the controls and 64% of the cells in patients. B lymphocytes accounted for 19% of the circulating lymphocytes in control subjects and 12% in breast cancer patients and this difference was

Fig. 42

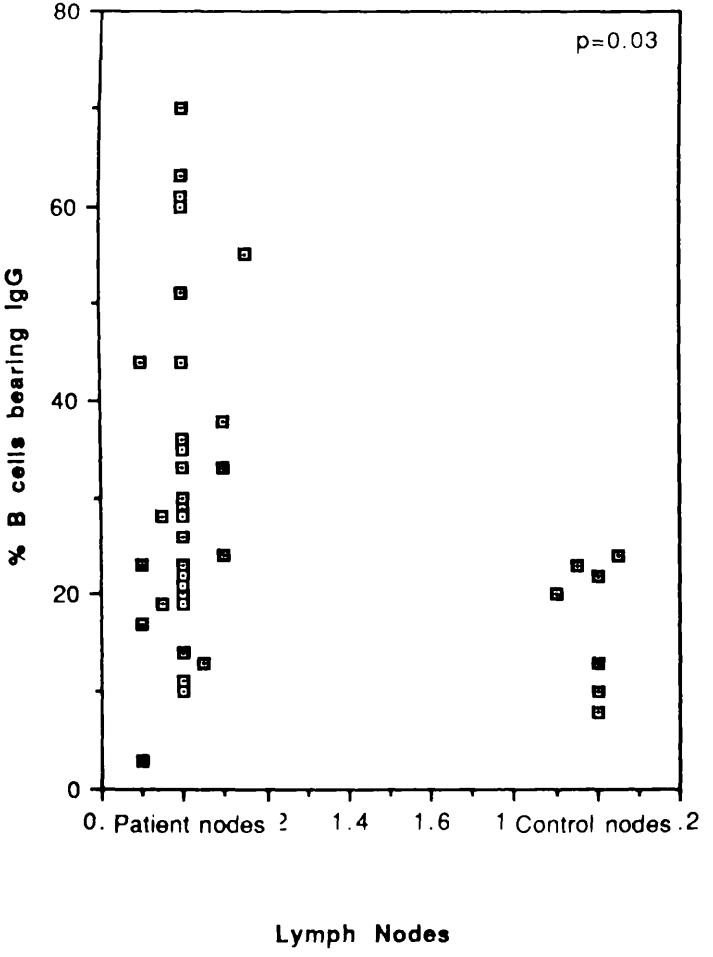
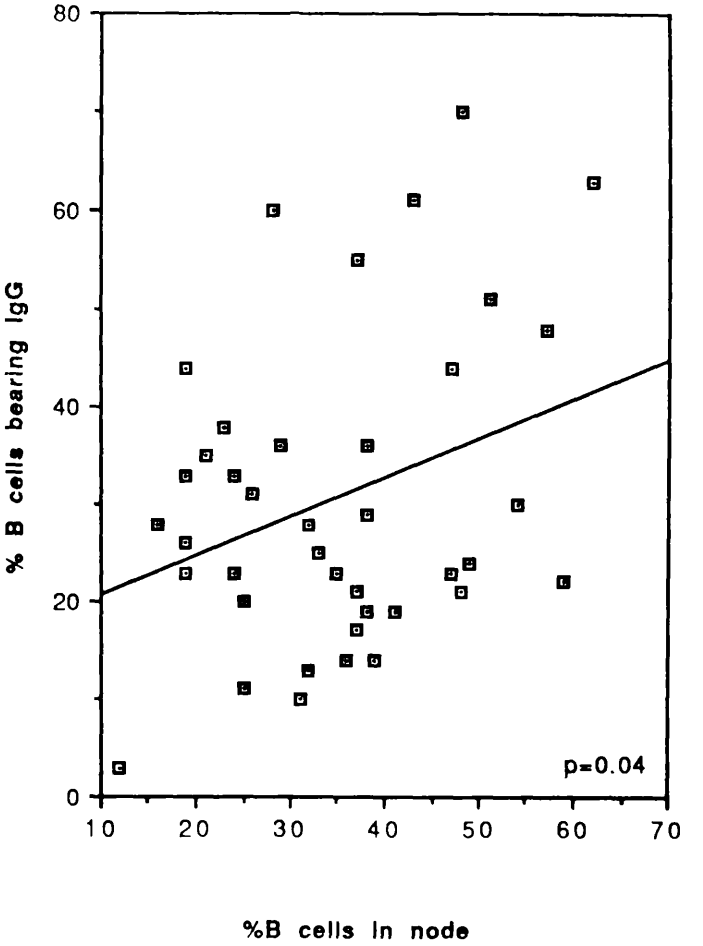

p=0.03
% B cells bearing IgG
80
60
40
20
0
0. Patient nodes
1.4
1.6
1 Control nodes
.2
Lymph Nodes

Fig. 43


% B cells bearing IgG
80
60
40
20
0
10
20
30
40
50
60
70
p=0.04
%B cells in node

statistically significant (p=0.006) (Fig. 44). Further subdivision of the T lymphocytes into CD4+ helper T cells and CD8+ suppressor/cytotoxic T cells revealed that, on average, 40% of the T cells in normal subjects were of the CD4+ phenotype while 24% were of the CD8+ phenotype with a helper/cytotoxic ratio of 1.7 (Table 7). The number of CD4+ and CD8+ cells in the control samples also fell within limited ranges of 30% to 54% and 16% to 30% respectively with a ratio ranging from 1.07 to 2.44. There was much greater variation among the cancer patients with the percentage of CD4+ helper T cells ranging from 8% to 70% and the CD8+ suppressor/cytotoxic cells ranging from 6% to 47%. This gives a CD4+/CD8+ ratio which itself ranges from 0.53 to 5.00 (Fig. 45) although the mean size of each subset and the average ratio were similar to those of the controls.

ACTIVATION MARKERS

HLA DR

This activation marker was found on many more T cells in the breast cancer patients, being present on 36% of CD8+ suppressor/cytotoxic T cells as compared to only 14% of normal CD8+ cells ($p<0.0001$) (Fig. 46a) while for the

PARAMETER	PATIENT BLOOD n = 39	CONTROL BLOOD n = 7	STUDENT'S t TEST
% T cells	64.4 ± 2.33	58.9 ± 4.88	t = 0.943 p = 0.351
% B cells	12.2 ± 0.87	19.4 ± 3.48	t = -2.911 p = 0.006
% CD4+ T cells	39.9 ± 1.99	40.3 ± 3.16	t = -0.079 p = 0.937
% CD8+ T cells	26.2 ± 1.52	26.2 ± 1.52	t = 0.544 p = 0.589
CD4+/CD8+ ratio	1.7 ± 0.14	1.7 ± 0.18	t = 0.016 p = 0.988

Table 7 Comparison of the phenotypic proportions in peripheral blood from breast cancer patients and normal subjects.

Fig. 44

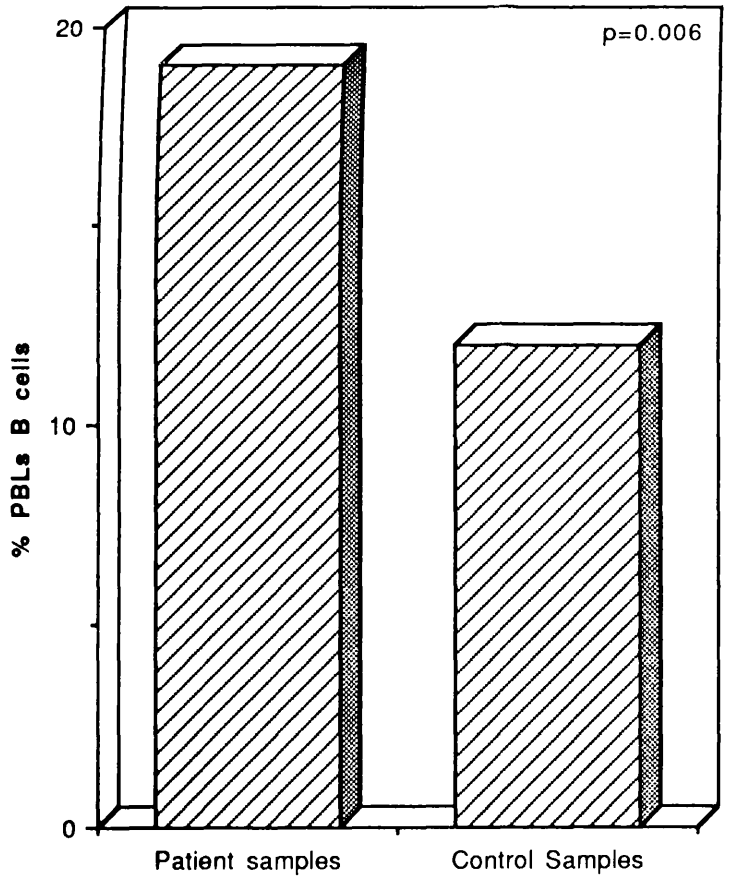

20
10
0
p=0.006
% PBLs B cells
Patient samples
Control Samples
Peripheral Blood

Fig. 45

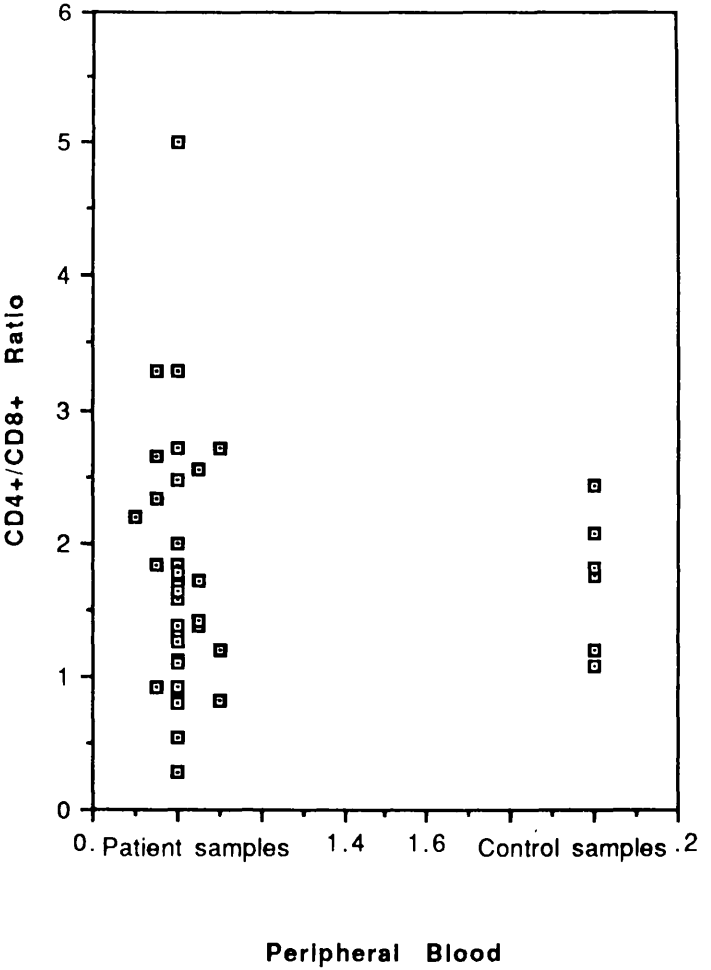

6
5
4
3
2
1
0
CD4+/CD8+ Ratio
0.
Patient samples
1.4
1.6
Control samples
.2
Peripheral Blood

Fig. 46a

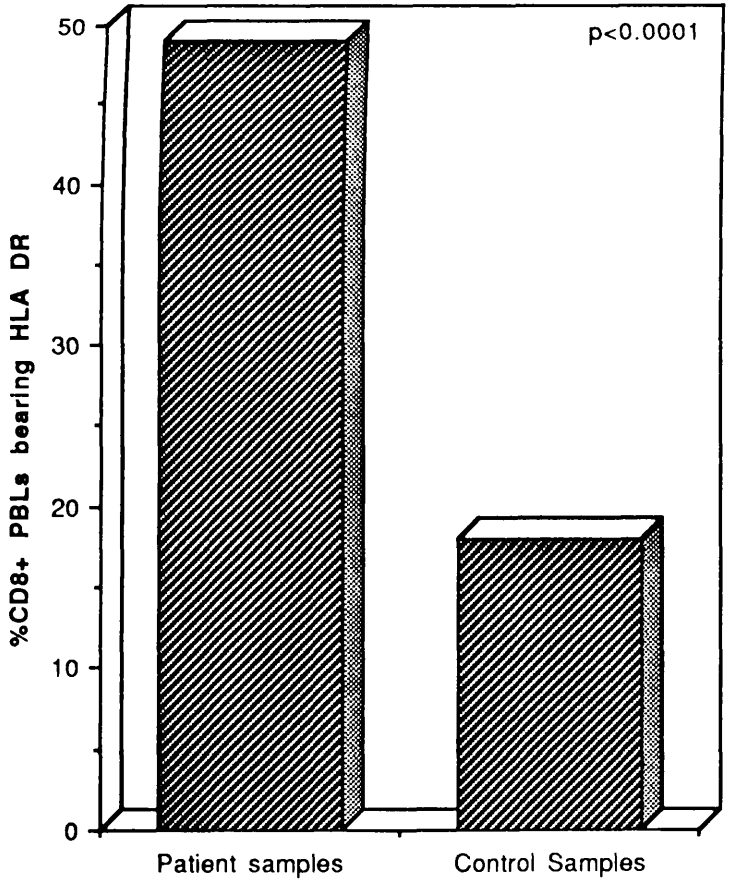
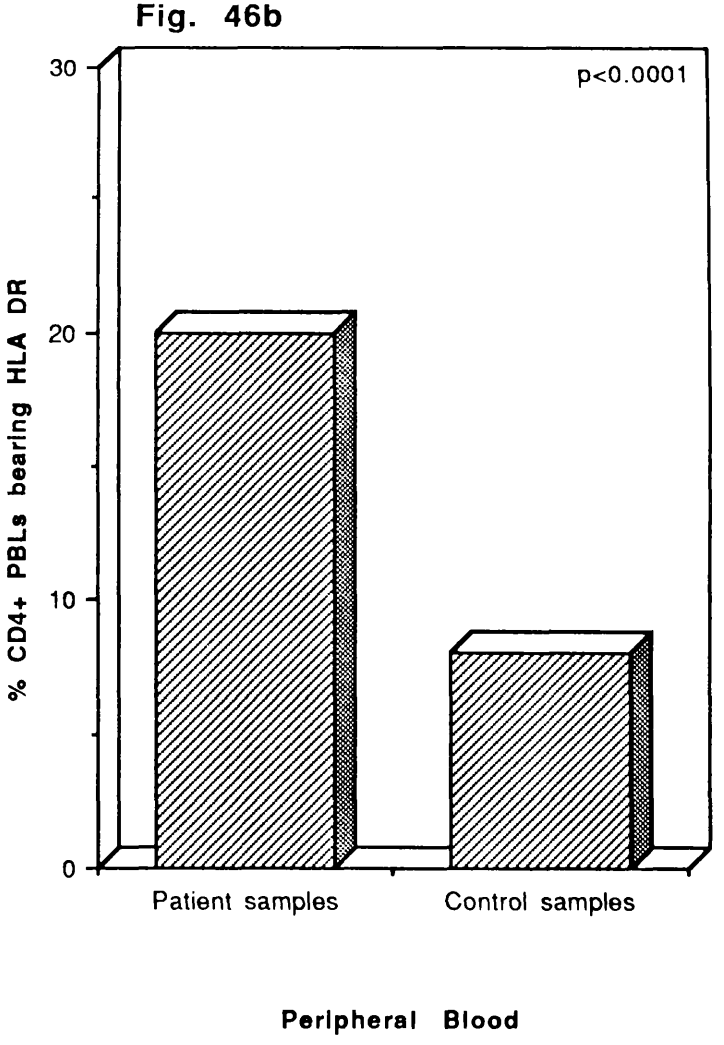


Fig. 46b



CD4+ helper T cells the proportions were 19% and 8% respectively ($p<0.0001$) (Table 8; Fig. 46b).
HLA DR was found, as expected, on virtually all B lymphocytes. No relationship between the expression of this marker and disease stage was identified (Table 9).

Interleukin 2 receptor

This surface antigen was also present on greater numbers of T lymphocytes in the patient samples. 15% of the patients' CD8+ T cells bore the Tac antigen while 9% of the control CD8+ T cells did so ($p<0.03$) (Fig. 47a) and while 31% of the patients' CD4+ T cells were positive only 20% of the control CD4+ cells were so ($p=0.006$) (Fig. 47b).
The number of B lymphocytes bearing this marker was similar in each group with 21% of patients' and 18% of the control B cells positive for Tac. In all samples, from both normal subjects and breast cancer patients, the Tac antigen was found on more CD4+ T cells than CD8+ T cells ($p<0.0001$) (Fig. 48). This marker also did not appear to be affected by tumour stage.

Transferrin receptor

This marker was carried by slightly more cells, of all phenotypes, in the peripheral blood samples of patients with breast carcinoma than in those from normal subjects

PARAMETER	PATIENT BLOOD n = 39	CONTROL BLOOD n = 7	STUDENT'S t TEST
CD8+ T cells bearing HLA DR	35.6 ± 1.70	13.9 ± 1.78	t = 5.276 p < 0.0001
CD8+ T cells bearing Tac	15.1 ± 1.05	8.9 ± 2.28	t = 2.348 p = 0.023
CD8+ T cells bearing TrfR	37.9 ± 1.97	23.3 ± 10.35	t = 1.926 p = 0.061
CD4+ T cells bearing HLA DR	19.1 ± 1.07	8.1 ± 0.80	t = 4.311 p < 0.0001
CD4+ T cells bearing Tac	30.5 ± 1.47	20.1 ± 1.58	t = 2.920 p = 0.006
CD4+ T cells bearing TrfR	35.0 ± 2.08	25.3 ± 10.59	t = 1.215 p = 0.23
B cells with high HLA DR	9.6 ± 1.33	25.3 ± 7.26	t = -3.644 p = 0.001
B cells bearing Tac	21.7 ± 1.81	18.1 ± 4.65	t = 0.756 p = 0.454
B cells bearing TrfR	49.1 ± 2.04	54.3 ± 10.33	t = -1.478 p = 0.147
B cells bearing surface IgG	17.7 ± 1.43	6.9 ± 1.61	t = 3.172 p = 0.003

Table 8 Comparing the expression of activation markers on the peripheral blood lymphocytes of patients with breast cancer and normal subjects.

PARAMETER PBLs (n = 39)	CORRELATION WITH TUMOUR STAGE (Spearman Rank correlation)		CORRELATION GRADIENT
% T cells	r = 0.312	p = 0.157	NS
% B cells	r = -0.073	p = 0.746	NS
% CD4+ T cells	r = 0.239	p = 0.284	NS
% CD8+ T cells	r = 0.160	p = 0.478	NS
CD4+/CD8+	r = 0.219	p = 0.327	NS
%CD8+ HLA DR+	r = 0.033	p = 0.884	NS
%CD8+ Tac+	r = -0.146	p = 0.515	NS
%CD8+ TrfR+	r = -0.126	p = 0.576	NS
%CD4+ HLA DR+	r = 0.119	p = 0.606	NS
%CD4+ Tac+	r = -0.013	p = 0.953	NS
%CD4+ TrfR+	r = 0.146	p = 0.515	NS
%B high HLA DR	r = -0.001	p = 0.996	NS
%B Tac+	r = 0.329	p = 0.146	NS
%B TrfR+	r = 0.086	p = 0.702	NS
%B IgG+	r = -0.020	p = 0.930	NS

Table 9 Correlation of the surface markers borne by peripheral blood lymphocytes with disease stage. There is no change in the activation status of the peripheral blood lymphocytes with advancing tumour spread.

Fig. 47a

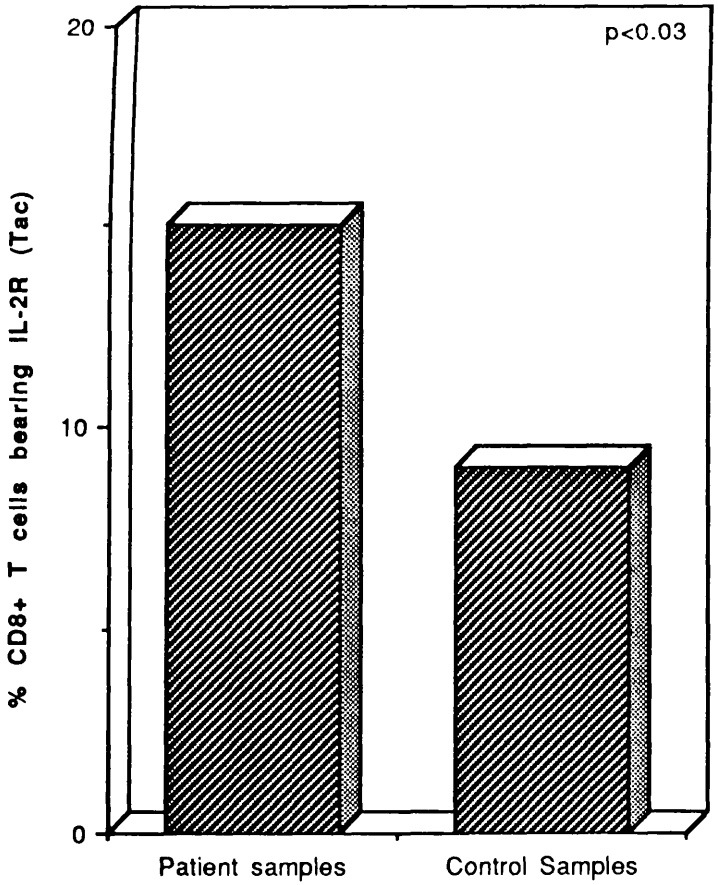


Fig. 47b

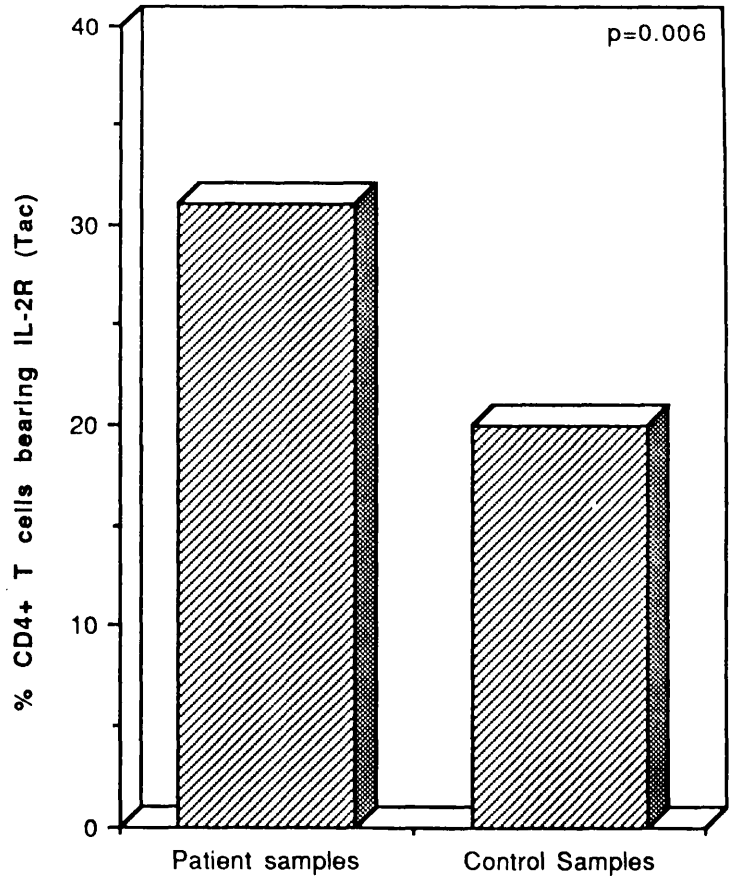
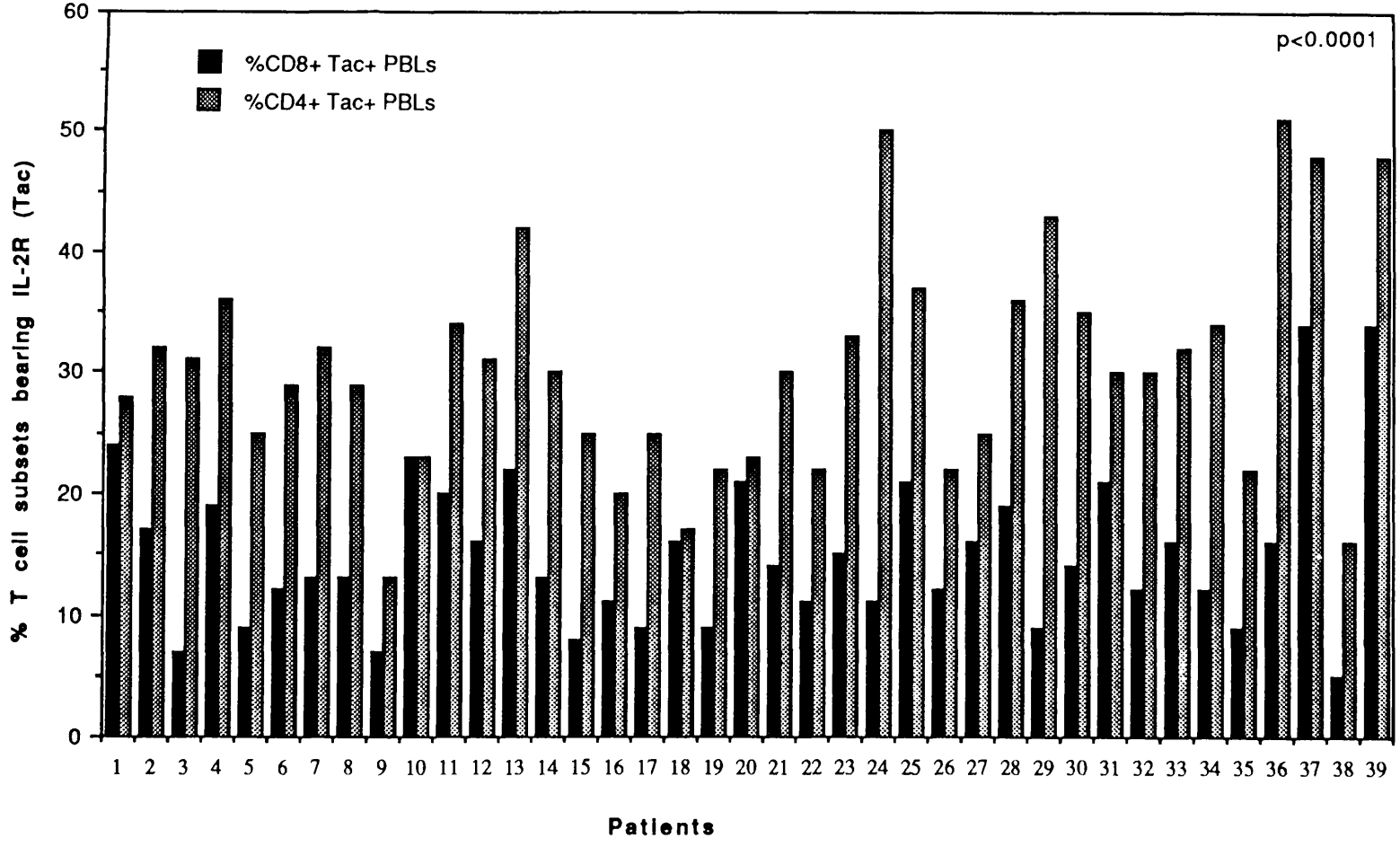


Fig. 48

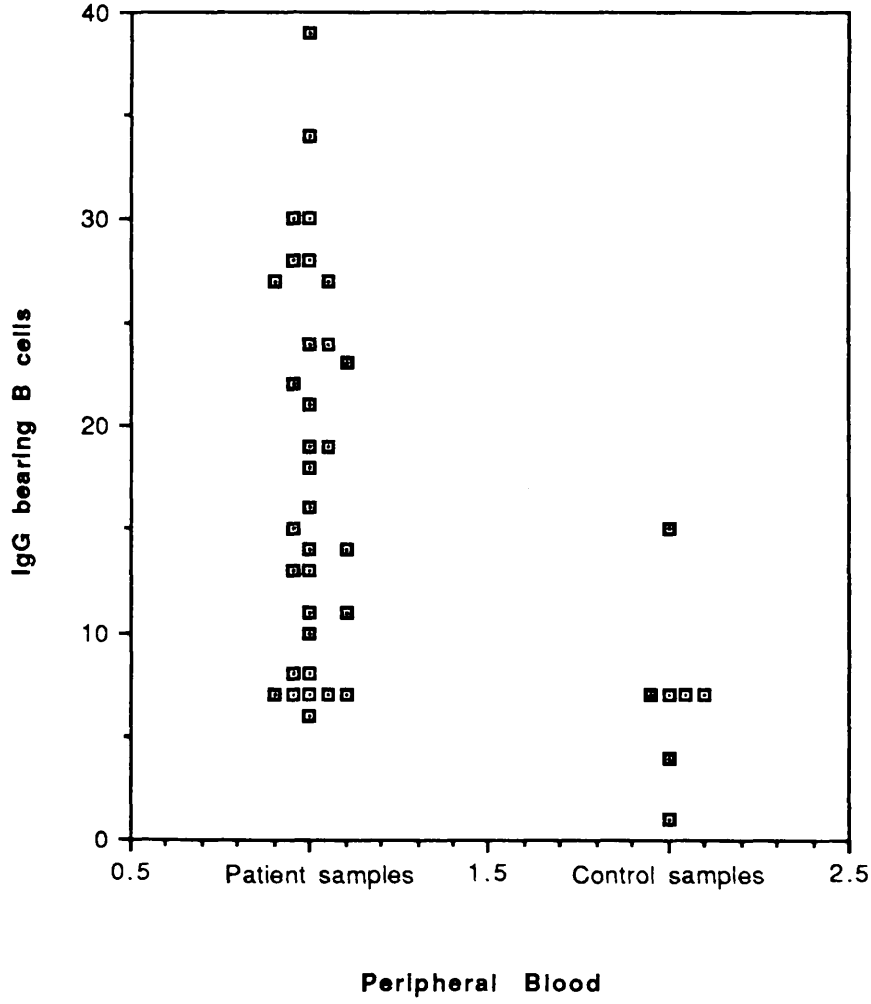

p<0.0001
%CD8+ Tac+ PBLs
%CD4+ Tac+ PBLs
% T cell subsets bearing IL-2R (Tac)
0
10
20
30
40
50
60
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
Patients

but this was not statistically significant.

Immunoglobulin G

There were greater numbers of IgG committed B lymphocytes circulating in the blood of breast cancer patients than in normal controls. In the breast cancer patients an average of 18% of B cells bore surface IgG as compared to only 7% in the control group ($p=0.003$) (Fig. 49).

Fig. 49


40
30
20
10
0
IgG bearing B cells
0.5
Patient samples
1.5
Control samples
2.5
Peripheral Blood

DISCUSSION

TUMOUR INFILTRATING LYMPHOCYTES

PHENOTYPIC MARKERS

From these results it can be seen that about 85% of the tumours studied had enough infiltrating lymphocytes to allow cell phenotyping to be carried out with 60% having sufficient to also allow the activation markers to be studied. This is similar to the findings of other groups [21, 137]. In this study, the maximum infiltrate accounted for slightly more than 80% of the cells harvested from the tumour and the highest ratio of lymphocytes to tumour cells was therefore 4:1. This calls into question the use, in cytotoxicity assays, of effector/target ratios as high as 50:1.

Like other groups, we found the infiltrate to consist largely of T lymphocytes. We found CD8+ suppressor/cytotoxic T cells to predominate in most tumours which is in agreement with the previous work by Giorno [159], Bhan & DesMarais [29], Rowe & Beverly [362], Hurlimann & Saraga [204], Lwin *et al* [261], Whiteside *et al* [459], Tanaka *et al* [410], Bilik *et al* [30] and Naukkarinen & Syrjänen [300], although at odds with the findings of

other groups such as Whitwell *et al* [463], Göttlinger *et al* [164], Horny & Horst [193], Ben-Ezra & Sheibani [25], Underwood *et al* [435], Zuk & Walker [476], von Kleist *et al* [233], An *et al* [7] and Balch *et al* [15], who found a predominance of CD4+ helper T cells. The widely conflicting phenotypic proportions found within breast tumours by different groups may reflect the different methods used. Most groups have used tissue sections stained with monoclonal antibodies to the phenotypic markers which were conjugated to either an enzyme or a fluorochrome. It is therefore possible that sample variability may have played a part in giving such a variety of results. This is particularly important in view of tumour heterogeneity [187, 112], the effect of which is reduced by processing larger amounts of tumour. It was however notable that while the CD8+ suppressor/cytotoxic T cells predominated in most tumours, CD4+ helper T cells were seen in large numbers in tumours with a strong infiltrate. This pattern has been noted previously in other studies [193, 300] and it may therefore be that patient selection is actually responsible for the conflicting results. If only those tumours with a sizable infiltrate are studied, more are seen with a large CD4+ T cell infiltrate whereas if tumours are selected at random and even those with very few lymphocytes are characterized the picture alters in

favour of CD8+ T cell infiltration.

Like others [193, 475], we did not find a strong relationship between the degree of lymphocytic infiltrate and tumour stage. Although one group found an increased infiltrate in tumours which had metastasized to the axilla [233], our results do not confirm this.

There appeared to be a slight inverse correlation with the presence of the oestrogen receptor (ER) on the tumour cells, the ER negative tumours having greater lymphocytic infiltration, but this did not achieve statistical significance. Underwood *et al* [435] also did not find a statistically significant correlation between lack of ER and lymphocytic infiltration but this trend can be seen in their results and several other groups have found a similar inverse correlation which did achieve significance [204, 102, 7, 300]. The failure of the present study to demonstrate statistical significance may reflect the need for larger patient numbers.

This trend would also be in keeping with the finding that the degree of infiltration by CD8+ suppressor/cytotoxic T cells correlates strongly with histological tumour grade, suggesting that a lymphocytic reaction is more likely in tumours which are poorly differentiated. This finding suggests that the immune

system is able to detect tumour antigen on these undifferentiated and markedly abnormal cells and this is clearly of importance in the search to identify tumour antigen. Several other groups have also found some correlation between lymphocytic infiltration and tumour grade [475, 40, 30, 300], although they did not study the effect of tumour grade on T cell subset proportions. While no correlation with the presence of the class II antigen was seen, there was a fairly strong correlation between the degree of lymphocytic infiltrate and the expression of the class I antigen on the tumour cells. Again this relationship was most strongly seen with the infiltration of CD8+ T cells. As the CD8+ suppressor/cytotoxic T cells are known to recognize target antigens only in association with the class I MHC complex [165, 104, 268, 184], it is likely that antigen can only be detected and a response mounted when the class I complex is present. While Bhan & DesMarais [29], Whitwell *et al* [463], Zuk & Walker [476] and Möller *et al* [282] found no correlation between lymphocytic infiltrate and class I expression, our findings are in keeping with those of Rowe & Beverley [362], Hurlimann & Saraga [204], Del Giglio *et al* [100] and Nakazawa *et al* [297], who found a strong correlation in breast tumours and with others, who found a similar correlation in other malignancies [364, 255]. The reason for these conflicting results may

be due to the heterogeneous expression of these antigens on the tumour cells and the consequent sample variation. Two groups also found a correlation between the degree of infiltrate and the expression of the class II MHC antigen [418, 164] and this has been noted in other tumours [122] but while we also found a trend in this direction, it did not achieve statistical significance. Tumour cell expression of the class II MHC antigen was found to correlate with the activation status of cells within the tumour infiltrate rather than with its phenotypic composition.

ACTIVATION MARKERS

HLA DR

HLA DR is a marker of both T cell activation and antigen presentation [338] and is found on greatly increased numbers of peripheral blood CD8+ T cells and renal tubule cells of patients undergoing host rejection of a transplanted kidney [121, 184].

A similar distribution is seen in the breast cancer patients within this study where there is a correlation between the HLA DR expression on the tumour cells and the infiltrating lymphocytes.

The association with antigen presentation is also

supported by our finding that this marker is present on greater numbers of tumour infiltrating lymphocytes, particularly CD8+ T cells, with increasing histological grade and increasing tumour cell expression of the class I MHC complex suggesting that the immune system is able to detect antigen on the surface of cells from poorly differentiated tumours when presented alongside the class I MHC complex. This marker tends to be expressed on more CD8+ than CD4+ T cells [340] and is present on a greater number of CD8+ T cells as the proportion of these cells within the infiltrate increases. It is possible that, after recognition of the tumour antigen combined with the class I MHC complex and destruction of the tumour cell, the CD8+ cytotoxic cells present part of the antigenic structure of the "foreign" cell within the groove of their HLA DR complex to signal to other cells of the immune system.

While several groups have found HLA DR to be carried by an increased number of T cells infiltrating breast tumours [261, 25] and other malignancies [188, 186, 407, 307] there is a great variation in results, with those methods which involve long preparation times, or fixing of the cells, showing fewer positive cells. This is most notable in the study by Whiteside *et al* [460] where collagenase digestion, followed by a long incubation period, was used to release the lymphocytes from the

tumour prior to staining. This group found very few lymphocytes bearing any activation markers and it is likely that these were lost during the enzymatic digestion and incubation period.
As tumour cells also carry this surface antigen, staining with phenotypic antibodies is vital to ensure accurate assessment of the number of lymphocytes and tumour cells bearing this marker. This requires the use of dual immunofluorescent staining and fluorescent microscopy or flow cytometry. The relationship of the TIL expression of this activation marker to other features of the tumour, such as histological grade, has not previously been reported.

Interleukin 2 receptor

No great difference was found, in the number of lymphocytes bearing the IL-2 receptor, between tumours of differing grade or stage but it was most striking that consistently more of the CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells bore this marker. Some groups have found very few infiltrating lymphocytes bearing this marker in breast [460, 25] or other tumours [307, 407] but this may be due to the cell preparation as these groups used long incubation periods during cell harvesting or staining. They also did not use sodium azide to avoid capping of the antibody/antigen complexes

formed by the immunofluorescent antibody and the marker it stains. Studies performed using fresh cells [188, 186, 108] found a high proportion of IL-2 receptor bearing T cells while Lwin *et al* [261], using immunohistochemistry, found great variation between tumours. None of these groups looked at the distribution of cells bearing this receptor within the T cell subsets.

In this study we found the presence of the IL-2 receptor on the CD4+ T cells to correlate with both the extent of infiltration by these cells and the tumour cell expression of the class I and class II MHC antigens. This receptor only increases on cells after they have been stimulated by antigen and so its presence again suggests that these lymphocytes are recognizing some tumour antigen alongside the class I MHC complex. This receptor is a marker of cell division and this finding indicates increased turnover and possibly expansion of the CD4+ helper T cell subset. The CD8+ T cells show little evidence of division and this may be due to their terminal differentiation into functional cytotoxic cells with no further need of replication. The most notable finding was the consistent presence of this receptor on almost twice as many CD4+ helper T cells as CD8+ suppressor/cytotoxic T cells. This may be due to greater activation of the CD4+ T cells or may

represent a functional difference between the two T cell subsets with regard to stimulation by IL-2.
This is of importance as, in TIL therapy, the TILs are harvested and cultured in IL-2 before being returned to the patient. The rationale for this therapy is that the cytotoxic cells, which have already been primed by tumour antigen, will greatly increase in numbers in the IL-2 culture and, upon return to the patient, will destroy metastatic deposits of tumour [354]. Two groups, who phenotyped the TILs from malignant melanoma before IL-2 expansion, also found the IL-2 receptor to be present on more of the CD4+ T cells than the CD8+ T cells [258, 15]. Although some groups, who phenotyped the cells after IL-2 culture, found CD8+ T cells to predominate [247, 427], others found the greatest expansion to be in the CD4+ helper T cell population, with the CD8+ suppressor/cytotoxic T cells dying away after two or three weeks. Thus, the cells being returned to the patient, as TIL therapy, were predominantly of the helper T cell phenotype [22, 294, 185, 242, 277, 356, 23, 386]. As TIL therapy has produced some striking responses, particularly in malignant melanoma [354, 357], it is possible that these have actually been mediated by the CD4+ helper T cells rather than the remaining CD8+ T cells. How these responses have been mediated must be clarified to allow

further development of this therapeutic approach.

Transferrin receptor

Increased transferrin receptor levels on the membrane of a cell suggest it is preparing for division at which time the iron requirement increases because of the production of ribonucleotide reductase. This marker was found on a greater number of CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells again suggesting there is more cell division in this subset. No relationship was found between this marker and the prognostic factors or tumour cell expression of the class I or class II MHC antigens.

Immunolglobulin G

A great variation was seen in the number of tumour cells with IgG bound to their surface membrane. The distribution of this immunoglobulin within the tumours could not be further clarified by flow cytometry but several studies, using immunohistochemical techniques, have also found IgG to be the major immunoglobulin and have found the greatest concentration to be in the tumour stroma [194, 106, 208].

The presence of tumour bound IgG did not relate to tumour stage, grade or ER status but showed some correlation with expression of the class I MHC complex

on the tumour cells. Although we found no relationship between the presence of the ER and IgG on the tumour cells, an inverse correlation has been noted by others [262, 465] and the lack of significance in our results may simply reflect the small number of tumours in which this parameter was studied. Roberts *et al* [348] noted a correlation between the amount of IgG in the tumour and the degree of lymphocytic infiltrate and whilst this trend was also seen in our results, it again did not achieve statistical significance. The effect of this membrane bound immunoglobulin on the tumour cells is not clear and while it is indicative of a humoral anti-tumour immune response there is evidence to suggest that in some patients this response is flawed. These antibodies may act as a shield, by blocking the antigenic sites, while not themselves activating a complement cascade or chemotaxis to bring about destruction of the antigenic tumour cell [183, 262, 107].

LYMPH NODE LYMPHOCYTES

It is clear from these results that there are major alterations of both the phenotypic proportions and the expression of surface activation markers in the axillary lymph nodes of patients with breast cancer.

PHENOTYPIC MARKERS

The grossly engorged appearance of some of the axillary lymph nodes from the breast cancer patients has been previously noted [34, 35, 94, 118] and contrasts sharply with the small pale control nodes and these appearances are reflected in the higher cell counts obtained from the former.

No difference was found in the proportion of T cells or B cells in the lymph nodes of breast cancer patients and normal controls. This is contrary to the findings of Eremin *et al* [118] who found an increase in the B cell population and a corresponding decrease in the T cell population in cancer patients but is in keeping with the findings of Heidenreich *et al* [182], Gupta *et al* [171] and Bonilla *et al* [50] who also found no difference between the two groups. Tsakraklides *et al* [430], Eremin *et al* [118] and Morton *et al* [289] found an increase in the B lymphocyte population in patients with stage II breast cancer and while we also detected a slight trend in this direction it did not achieve statistical significance.

In this study we found an overall increase in the CD4+/CD8+ ratio in the lymph nodes of cancer patients, due largely to a marked increase in the number of CD4+ helper T cells. There is also a much wider range of ratios among the cancer patients while normal ratios

range around 2:1, which is in keeping with other studies of normal nodes [195]. There are two other studies comparing the T cell subsets in breast cancer patients with those in normal controls [224, 272], with the former using immunohistochemical techniques while the latter used single colour flow cytometry. Both studies were small, having 8 patients each and Khuri *et al* [224] included only 3 control subjects. While Mantovani *et al* [272] found no difference in the overall phenotypic proportions of nodes from breast cancer patients and control subjects, Khuri *et al* [224] suggested there was an increase in the CD8+ T cell population in the cancer patients. Another group, who compared the phenotypic proportions of PBLs and LNLs in patients with breast cancer found a large proportion of CD4+ T cells in the nodes but, lacking any controls were unable to clarify whether this was due to the presence of malignant disease or merely to the lymphocyte source [289]. From our results it can be seen that this large CD4+ population occurs only in the cancer patients and is therefore likely to be part of an immune reaction to the tumour.

We, like Morton *et al* [289], found some decrease in the size of this population with advancing disease but it was not statistically significant while Mantovani *et al* [272] found an increase in the CD4+ population in nodes

containing metastatic deposits. Farzad *et al* [124], working with skin melanoma, and Cozzolino *et al* [83], working with tumours of the larynx and bladder, also noted a decrease in the CD4+ T cell population in patients with stage II disease.

From the wide variation in this population it can be seen that some patients have a smaller CD4+ T cell population than normal subjects along with a lower CD4+/CD8+ ratio. As this ratio is considered a useful guide to immune competence in patients with immunosuppressive conditions such as AIDS [236], it may also be a useful indicator of immunosuppression or stimulation of the immune system, by the tumour, in patients with breast cancer.

ACTIVATION MARKERS

HLA DR

In this study we found this marker to be present in similar quantities on B lymphocytes from the two groups but on almost treble the number of T cells, of both phenotypes, from the axillary nodes of breast cancer patients compared with normal controls. Only one other study has been performed comparing the expression of this marker in the nodes of normal subjects and breast

cancer patients [272] and, as this was carried out using single colour flow cytometry, the phenotypic distribution of the HLA DR was not demonstrated. Mantovani *et al* [272] found no significant difference, in the expression of this marker, between the LNLs of the two groups but the source of the control nodes is not stated and, as they were found to have a fairly high proportion of HLA DR bearing lymphocytes, it is possible that they were excised because of a benign disease process which might nevertheless have caused some degree of immune stimulation.

This marker of activation and antigen presentation was expressed on a greater proportion of the CD8+ T cell population in patients with stage II disease. As these patients had nodal metastases, the CD8+ T cells within the nodes were again in direct contact with tumour cells and tumour antigen, which may have lead to their stimulation. Morton *et al* [289] also found an increase in the number of cells bearing this marker in stage II disease but Mantovani *et al* [272], comparing invaded and uninvaded nodes, found the expression of HLA DR to be higher in the uninvaded nodes. Two groups, working with other tumours, found no relationship between HLA DR expression and tumour stage [124, 83].

Interleukin 2 receptor

While this marker was unaltered in the B cell population it was present on many more T cells in the lymph nodes of patients with breast cancer. Again this increase was seen in both subsets with the greatest number of positive cells in the CD4+ subgroup. Tac, which is the 55Kd component of the interleukin 2 (IL-2) receptor, is present at very low levels on resting T cells but greatly increases on those which have been stimulated through the T cell antigen receptor complex [446, 387]. After stimulation by antigen or mitogen, the number of surface receptors increase in the presence of IL-2. The presence of this receptor on greater numbers of both T cell subsets in the lymph nodes of breast cancer patients suggests that they have been stimulated by antigen and are being maintained by the presence of IL-2. There are no other studies in this field which compare the expression of the Tac marker on lymphocytes from the lymph nodes of breast cancer patients with those from normal subjects but it was studied by Cozzolino *et al* [83] who worked with laryngeal and bladder carcinoma and Farzad *et al* [124] who worked with skin melanoma. While Farzad *et al* [124] found this receptor on almost no cells in the regional lymph nodes of patients with skin melanoma, Cozzolino *et al* [83] found it on up to 30% of LNLs with a mean of 21% in nodes with metastatic

tumour and 12% in nodes uninvolved with tumour. The difference in the findings of Farzad *et al* [124] and both Cozzolino *et al* [83] and ourselves may be due to the long incubation period used when staining the cells with monoclonal antibodies. They were maintained overnight at 4°C without the presence of sodium azide, and this may have allowed the antibody/antigen complexes to "cap" and become internalised thus greatly reducing the surface antibody remaining to be detected by flow cytometry.

Unlike Cozzolino *et al* [83], we found no correlation between the size of the IL-2 receptor bearing population and tumour stage.

Transferrin receptor

Although we found a slight increase in the number of cells bearing this receptor in the nodes of the cancer patients it was not statistically significant and showed no relationship to tumour stage. This marker has not been studied on lymph node lymphocytes by other groups. Although the expression of this receptor usually increases after IL-2 stimulation and just prior to cell division, there is some evidence to suggest that a second pathway exists through which IL-2 stimulation leads directly to cell division without a major increase in the number of transferrin receptors [432].

Immunoglobulin G

More IgG committed B lymphocytes were detected in the axillary lymph nodes of the breast cancer patients than in the normal nodes. A broad range of IgG commitment was found in the cancer patients while in the controls the level fell within a much tighter band suggesting that there may be a normal level of IgG bearing B cells and that some breast cancer patients fall below this while others show evidence of raised IgG production. Artifactual causes for this apparent increase were avoided by the use of double staining with the CD19 marker antibody so that only cells of the B lymphocyte phenotype were studied. Other disease conditions which might cause such a response had been excluded at the time of patient selection.

Richters & Kaspersky [343] were the first to study the surface immunoglobulins on B lymphocytes from the axillary lymph nodes of patients with breast cancer and they found IgG to be carried by 22% of B cells with a range from 2% to 50%. They did not include any normal controls but Eremin *et al* [118] compared the surface immunoglobulins borne on lymphocytes from the axillary lymph nodes of patients with those from control subjects. They also found the normal level of IgG bearing B cells in lymph nodes to be about 17% while in breast cancer patients it was about 28%. This presence

of large numbers of B cells bearing IgG suggests a mature secondary humoral immune response [351]. Eremin *et al* [118], like us, found no alteration with tumour stage but one other group, using histochemical analysis of immunoglobulin distribution, found more IgG bearing B cells in the nodes of patients with stage II disease and therefore identified this as a bad prognostic indicator [439].

PERIPHERAL BLOOD

PHENOTYPIC MARKERS

In contrast to other studies comparing the peripheral blood of breast cancer patients and controls, we found a slight fall in the proportion of B lymphocytes. Previous studies have found either no difference [116, 205, 309, 260, 325] or a fall in the proportion of T lymphocytes [458, 222, 182] with the last group suggesting a compensatory rise in the B cell population. One group [359] suggested a rise in the peripheral T cell population was associated with a poor prognosis.
The apparent fall in the number of B cells detected in this study could reflect the maturity of these cells as the B cell phenotype marker is lost when they become

terminally differentiated plasma cells.

The T cell population was further divided into the helper and suppressor/cytotoxic subsets and while the average proportions and ratio were almost identical and fell within accepted normal ranges [452, 161, 375, 269], the variation in phenotypic subgroup proportions was far greater in the cancer patients. This suggests that while the control samples may represent "normal" phenotypic proportions, the cancer patients appear to be scattered to the extremes which may be due to suppression or stimulation of the immune system. Mantovani *et al* [272] also found no difference in the phenotypic proportions between breast cancer patients and controls but great variation in the CD4+/CD8+ ratios was noted by another group who used flow cytometry [378]. This variation, combined with patient selection may explain the differences between our findings and those of Pattanapanyasat *et al* [325] who found the cancer patients to have a larger CD8+ T cell population and Valavaara *et al* [440] who, studying the response to anti-oestrogen therapy, found them to have a smaller CD4+ T cell population prior to treatment.

A broad range of CD4+/CD8+ ratios was not noted by groups using fluorescent microscopy to study patients undergoing postoperative radiotherapy or chemotherapy who also found initial CD4+/CD8+ ratios to be within the

normal range [330, 331, 425, 332, 305]. The ratios later fell, due to a selective loss of the CD4+ helper T cells as the course of adjuvant therapy progressed. The use of microscopy is limited in this area by the difficulty of counting sufficiently large numbers of cells to give statistical accuracy and the group who used flow cytometry only counted 1000 cells for each sample. Apart from the technical differences, in all these studies the initial samples were taken after surgery which may itself cause some disturbance of the immune system [77].

High CD4+/CD8+ ratios have been suggested as a sign of immunocompetence in patients with immunosuppressive disorders [236] and low ratios are certainly found in patients with immunosuppression secondary to chemotherapy [332, 331, 378]. The CD4+/CD8+ T cell ratio of the peripheral blood lymphocytes may therefore act as a useful guide to the immune status of patients with breast cancer or to immunosuppression in patients undergoing adjuvant therapy.

ACTIVATION MARKERS

HLA DR

This marker of activation and antigen presentation in T lymphocytes, was present on greater numbers of both helper and suppressor/cytotoxic T cells in the peripheral blood of breast cancer patients. We found this marker to be present on greater numbers of T cells than Pattanapanyasat *et al* [325] but our figures are similar to those of Mantovani *et al* [272] who, using this as their only activation marker, found it to be present on about treble the number of PBLs in the cancer patients. As this group used only single colour flow cytometry they could not determine the cellular distribution of this marker. Lakhdar *et al* [249], in their study of patients with nasopharyngeal carcinoma, also found about 35% of the circulating T cells to be carrying this marker. It was found on a similar proportion of T cells by van Es *et al* [121] in patients with an allogenic kidney graft, suggesting its relationship with the chronic presence of antigenic material.

Interleukin 2 receptor

Little difference was seen in the numbers of B cells bearing this receptor in the two groups but there was a

significant increase in the number of T cells positive for this receptor and particularly striking was the finding that this receptor was again consistently present on more CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells, in both normal subjects and patients with breast cancer. While Pattanapanyasat *et al* [325] found no difference between the control and cancer patients with regard to this receptor, Lakhdar *et al* [249], in their study of patients with nasopharyngeal carcinoma, found a similar increase in the number of T cells bearing the IL-2 receptor. These groups did not study the distribution of this receptor within the T cell subsets.

The expression of this receptor on more of the CD4+ helper T cells may reflect a greater stimulation of these cells by antigen or may represent a constitutional difference between them and the CD8+ suppressor/cytotoxic T cells. As this pattern is also found among the normal subjects, the latter would appear more likely.

Transferrin receptor

As was the case with the lymph node lymphocytes, no significant difference was found in the number of cells bearing this receptor between the breast cancer patients and the normal controls. This is in keeping with the

findings of Pattanapanyasat *et al* [325].

Immunoglobulin G

More IgG bearing B cells were found to be circulating in the breast cancer patients as opposed to the normal controls suggesting that there may be some degree of secondary or chronic humoral response in many of the patients. Several approaches have been taken to studying the humoral immune response within peripheral blood. Most groups have studied the serum immunoglobulins either directly [349, 441, 320] or by assaying the serum of patients against breast cancer cell lines [113, 200] or mouse mammary tumour virus (MMTV) [336, 426]. While studies using direct assay produced no clear evidence of a humoral immune response in breast cancer patients, those looking at the binding of patient sera on tumour sections, cell lines or against MMTV suggested there was circulating anti-tumour immunoglobulin. Most of these studies found no relationship between the presence of serum immunoglobulin and prognosis but one group, who studied both the serum levels of IgG and the proportion of IgG bearing B cells, found this to correlate with a poor outcome [474]. This would be in keeping with the theory of antigenic sites being shielded by antibodies which themselves fail to trigger an immune reaction [183, 107].

CONCLUSIONS

PRIMARY TUMOUR

85% of the breast tumours, in this study, show some evidence of a cellular immune response consisting of T lymphocytes and in 60% this is sufficient to allow detailed analysis. CD8+ suppressor/cytotoxic T cells predominate and increase with histological grade and the expression of the class I MHC complex on the tumour cells. This suggests that the first reaction may be of the CD8+ cytotoxic T cells to tumour antigen presented along with the class I MHC complex.

The expression of HLA DR on the CD8+ T cells also increases with tumour grade and expression of the class I MHC complex. It is possible that antigen is further presented by the CD8+ T cells to the CD4+ helper T cells which are only found in significant numbers in those tumours with a heavy CD8+ T cell infiltrate.

The HLA DR expression on the CD4+ T cells also increases in poorly differentiated tumours again reinforcing its association with tumour antigenicity.

The receptors for transferrin and interleukin 2 are present on a higher proportion of the CD4+ T cells suggesting that while the CD8+ T cells are probably

fairly static and differentiated there is greater cell division and expansion among the CD4+ T cell population. The finding that, in all samples, the IL-2 receptor was found on more CD4+ T cells than CD8+ T cells suggests that there may also be a functional difference between these two cell types with regard to stimulation by IL-2. There appears therefore to be evidence, within the primary lesions themselves, of a cellular immune response to tumour antigen in poorly differentiated tumours which have high levels of the class I MHC complex.

AXILLARY LYMPH NODES

When axillary lymph nodes from breast cancer patients are compared with iliac nodes from control subjects, those from the cancer patients are seen to be much larger and rather engorged in contrast to the small pale control nodes.

The increase, in many patients, in the number of B lymphocytes bearing surface IgG is in keeping with a well developed secondary humoral immune response and as these patients had no concomitant disease it is likely that this response is to the carcinoma.

There is also a much greater CD4+ T cell population in

the cancer nodes and this leads to an increase in the CD4+/CD8+ ratio.

More of the CD4+ and CD8+ T cells from the axillary nodes of breast cancer patients express HLA DR than from the control nodes and, in the case of the CD8+ T cells, this increases with tumour stage. The increase in HLA DR expression on the nodal CD8+ T cells in patients with stage II disease is in keeping with the role of this marker in antigen recognition as, in this circumstance, the CD8+ T cells are again in direct contact with tumour cells and tumour antigen.

The presence of the IL-2 receptor on many more T cells, of both phenotypes, suggests antigenic stimulation and the presence of secreted IL-2 within the lymph node environment of many patients with breast cancer. More CD4+ T cells than CD8+ T cells were noted to bear this marker.

From this study therefore, there is evidence of antigen recognition, stimulation and an immune response, both cellular and humoral, in the regional lymph nodes of patients with breast carcinoma.

PERIPHERAL BLOOD

From the results of this study it would appear that, while there are no major alterations in the overall phenotypic proportions, there is a small but significant decrease in the B cell population and a greater range of T cell subset proportions among the breast cancer patients when compared with the controls.

There is a marked increase in the number of T cells, of both phenotypes, bearing HLA DR and the IL-2 receptor both of which are associated with antigenic stimulation and cell activation.

Again more CD4+ T cells than CD8+ T cells were found to carry the IL-2 receptor and, as this was also the case in the control samples, it is likely that this represents a constitutional difference between the two cell types with regard to their response to IL-2.

There are more circulating B cells bearing surface IgG in the peripheral blood of the breast cancer patients than in that of the control subjects and this suggests a mature humoral immune response.

These findings suggest that there is some immune activition of the peripheral blood lymphocytes in patients with breast cancer.

CHAPTER 3: THE CONTRIBUTION OF THE AXILLARY LYMPH NODES TO THE IMMUNE RESPONSE IN PATIENTS WITH BREAST CANCER

INTRODUCTION

For the majority of patients, the treatment of breast cancer involves surgical excision of the primary lesion by lumpectomy or mastectomy, along with exploration and excision sampling of the ipsilateral axillary nodes. In many cases, particularly if the breast has been conserved or the disease is stage II, the patient will also receive loco-regional radiotherapy.

From the results given in the preceding chapter it would appear that some patients with breast carcinoma are detecting antigen on the surface of the tumour cells, along with the class I MHC complex, and mounting an immune response to it. It is therefore important to clarify the tissue sites involved in this response and, from this, how they might be affected by the treatment modalities commonly used. Various approaches have been used to assess the importance of the regional nodes in any immune response but their exact role and contribution is still not clear [323].

HISTORICAL REVIEW

HISTOLOGICAL STUDIES

The possibility that the axillary lymph nodes might be more than just filters was first raised by Halsted himself in 1898 [176] when he noticed that cell proliferation in the axillary lymph nodes of breast cancer patients was associated with a good prognosis. In 1906, Schindler [371] found that lymph node metastases were less common in the presence of sinus cell hyperplasia but it was Black *et al* in 1953 [34], who suggested that this represented a host-tumour immune response. Black's observations were confirmed by others [26, 27, 448, 273, 8, 383, 431], although the interpretation occasionally differed [26, 27]. Berg *et al* [26, 27] and Fisher *et al* [136, 140] both found sinus hyperplasia to be associated with nodal status but not with survival and Steele *et al* [392] found the macrophages within the lymph node sinuses to be relatively inactivated. Several other morphological patterns were identified on histological examination, ranging from paracortical expansion and germinal centre predominance to unstimulated or even lymphocyte depleted nodes [431]. Paracortical expansion, representing an increase in the T cell area within the nodes appeared to be associated

with a good prognosis [345, 403], while lymphocyte depletion was found in those patients who already showed signs of widely disseminated disease.
Some groups found germinal center predominance, which suggests a proliferation of B cells, to be associated with an intermediate or even good prognosis [431, 147], while others found this pattern correlated with a bad prognosis [203, 138, 140]. In his initial work with breast carcinoma, Tsakraklides *et al* [431] found this pattern to be associated with an intermediate prognosis while in his later study of ovarian cancer it correlated with a poor outlook [430]. It has been suggested that this pattern represents a humoral response of antibodies which coat the tumour but do not initiate any further response of the immune system and mask the antigenic sites from further immunodetection [183, 107].

CLINICAL STUDIES

It was the possible contribution of the axillary lymph nodes to the host defence against breast cancer that led to the vigorous debate over the surgical management of breast cancer, with traditionalists believing fervently in the efficacy of radical surgery [173] while others proposed more conservative surgery with preservation of

the axillary nodes. Much of the drive for change came from George Crile, a Cleveland surgeon who was convinced of the value of the regional lymph nodes in the defence against breast cancer and who, in his own surgical practice, performed only simple mastectomies and preserved the axillary nodes. Presenting his own figures, he demonstrated a significantly increased 5 year survival in patients with clinical stage I disease [87, 88, 89]. This was supported by Montgomery *et al* [283] who performed only biopsy excision on 31 patients with invasive breast cancer and reported 100% survival at five years. In contrast to this, Urban [436, 437] proposed that, far from reducing the surgical excision, it should be extended to include the internal mammary nodes of patients with tumours of the medial aspect of the breast. In a series of patients treated using his surgical approach, a European group showed a survival advantage for the subset of patients undergoing this extended surgery who had internal mammary lymph node metastases [248].

Destruction of the axillary lymph nodes by radiotherapy was also shown to be harmful by several groups, who found it to be associated with both an increased relapse rate [74] and poorer survival rate [398, 304, 368], but it must be borne in mind that radiation affects tissues other than the regional nodes and that variations in

total radiation dose may affect outcome regardless of how the axillary nodes themselves are treated [14]. In an attempt to clarify the situation, over the surgical management of breast cancer, the National Surgical Adjuvant Breast Project was set up [131] which was a multicentre trial comparing simple mastectomy, simple mastectomy plus radiotherapy and radical mastectomy in the treatment of clinical stage I disease. After 10 years of follow up there was no significant difference in survival or widespread metastatic disease between the three groups but there was a higher incidence of loco-regional recurrence in the simple mastectomy group. However, 40% of the patients who underwent radical mastectomy and were clinically thought to have stage I disease, where found to have nodal metastases. As this was a randomised trial it is reasonable to assume that a similar proportion of patients in the simple mastectomy group had also, in fact, stage II disease. Only 15% of these patients went on to present, within the 10 years of follow up, with axillary metastases while the other 25% remained well and apparently disease free. It was also noted that while 88% of patients in the radical mastectomy group with loco-regional recurrences went on to develop systemic disease only 58% of similar patients in the simple mastectomy group went on to develop distant

metastases. This could be due to the fact that local recurrence in the radical mastectomy group represented truly recurrent disease which, being related to the aggressiveness of the tumour, was associated with disseminated disease while local recurrence in the simple mastectomy group often merely represented residual disease. From these findings it would appear that many patients can bear occult residual tumour which appears to cause little in the way of symptoms or occasionally presents many years later when the patient has been subjected to severe stress such as illness or bereavement [20, 223, 372].

The question, of course, is why this happens, whether it is due to features of the host defences or the tumour aggressiveness or a combination of both. In this large study of 1,665 women no significant survival difference was seen between the three clinically stage I groups regardless of management. However, as almost half of each group had in fact stage II disease, it is not possible to exclude a survival advantage or disadvantage for either true stage by the use of any of these three treatment modalities.

The best prognostic indicator remains lymph node stage [48, 135] and as there is, as yet, no other satisfactory way of staging patients [95, 2, 41, 333, 151] it is not possible to study the impact of leaving the axilla

intact in true stage I patients.

ANIMAL STUDIES

To try and gain more information about the role of the regional lymph nodes in cancer patients, several studies using animal tumour models were carried out.
Mitchison [280, 281] showed that the regional lymph nodes in a tumour bearing animal could transfer adoptive immunity to a greater degree than remote lymph nodes, splenocytes or peripheral blood lymphocytes.
Considering the contribution of the regional lymph nodes to the defence of the tumour host, Crile [86] found a greater number of mice with widespread metastases among those who underwent radical amputation of their tumours including excision or irradiation of the regional lymph nodes. This effect was lost if the amputation was delayed until a late period in the growth of the tumour suggesting that the nodes might be of greatest importance in the initiation and early phase of an immune response. This finding was confirmed by other studies [111, 328].
Studying the contribution of the nodes to tumour resistance, one group found that excision of the regional lymph nodes prior to inoculation with tumour

resulted in twice as many tumour takes in the group with no regional lymph nodes as in those with their regional nodes intact [126]. Excision of the nodes up to 4 weeks after tumour inoculation also resulted in increased tumour takes from a second tumour challenge, with tumours developing in 81% of control mice, 54% of those who had their regional lymph nodes excised and only 38% of those who had their regional lymph nodes left intact [127]. Clearly some centralised immune response had developed within the first four weeks but this experiment suggested that a significant contribution was still being made by the regional lymph nodes.

This was supported by a further study which demonstrated an effect of the regional lymph nodes on tumour growth up to two months after excision of the primary lesion [129].

Five groups, in studies involving methylcholanthrene induced sarcomas, found evidence more suggestive of a centralised immune response. Andreini *et al* [12], measuring the weight of various lymphoid tissues in tumour bearing animals, found the greatest increase to be in the spleen.

Gardner & Rosen [150] found no effect of radical tumour and lymph node excision on a second tumour challenge. Similarly, Hammond & Rolley [179] found little effect if the regional lymph nodes were retained or excised with

the primary tumour. Although Bard [18] showed that regional lymph node lymphocytes could indeed be used to transfer immunity from tumour bearing animals to syngeneic hosts as efficiently as splenocytes, he also found that while removal of the spleen significantly reduced the animal's resistance to a second tumour inoculum, excision of the regional lymph nodes had little effect. Humphrey *et al* [199] found that adding splenocytes from an immunized animal reduced successful tumour takes to 59% while 85% of the tumour inocula grew if combined with lymphocytes from the regional lymph nodes.

From these experiments it was concluded that regional lymph nodes were not necessary for an animal to mount an immune response against a tumour as splenocytes were just as effective, if not more so, in transferring immunity to other animals and the spleen appeared to be more important as a seat of immune memory for mounting a secondary immune response to any subsequent tumour challenge.

However, all of these studies were performed using chemically induced tumours which are known to be strongly antigenic [311] and Fisher & Fisher [127] found that chemically induced tumours grew significantly less often in previously immunized mice, whether the regional lymph nodes had been removed or not, whereas the lymph

nodes appeared to have a greater impact on the growth of spontaneous tumours. This suggested a different pattern of immune response in spontaneous and chemically induced tumours and studies of tumour models, which more closely resembled the poorly antigenic human tumours, revealed a weak immune response but with the regional lymph nodes making a greater contribution [335].

Unfortunately, because of these variations, the relevance of animal studies to patients is limited and many of the conflicting results are probably due to the use of different animal and tumour models.

FUNCTIONAL *IN VITRO* STUDIES

These experiments were performed using cells harvested from patients with breast cancer. Most groups compared lymph node lymphocytes with those from peripheral blood although some groups worked only with blood because of the ease of harvesting samples. The experiments fell into three main categories: blastogenesis assays, cytotoxicity assays and observations of *in vitro* behaviour such as cell migration or clumping.

BLASTOGENESIS ASSAYS

Using similar methods to those outlined in chapter 2, most groups found lymph node lymphocytes to have a higher thymidine uptake after mitogen stimulation than blood lymphocytes [103, 128], although Fisher *et al* [128] found the reverse when they used concentrated PHA. Reiss *et al* [339] found a higher cell turnover of lymph node lymphocytes from patients with small tumours and of peripheral blood lymphocytes from patients with large tumours. In a later study Fisher *et al* [130] found a greater cell turnover, after PHA stimulation, in lymphocytes from low axillary nodes than from high axillary nodes. The results of these experiments were variable but tended to suggest a greater lympho-proliferative response, to mitogenic stimulation, by lymph node lymphocytes than peripheral blood lymphocytes.

CYTOTOXICITY ASSAYS

These were again performed using the chromium release assay outlined in chapter 2.
Initially, the lymphocytes studied using this method were harvested either from the peripheral blood or

regional lymph nodes but later studies also looked at the cytotoxic effect of tumour infiltrating lymphocytes (TILs). As it is difficult to get fresh tumour cells to grow well in culture and take up the chromium, few studies have been carried out with autologous tumour cells as the target, although this is clearly the most relevant.

Using this approach, Hickok *et al* [191] found the lymph node lymphocytes (LNLs) to have a much greater cytotoxic effect on autologous tumour cells than peripheral blood lymphocytes. This reactivity could be transferred to peripheral blood lymphocytes by macrophages from hyperplastic nodes but not by those from small quiescent nodes. Skornick *et al* [386] found LNLs and TILs to have a similar degree of cytotoxicity against autologous tumour and found this to be greater than that of the PBLs. Belldegrun *et al* [24] found cytotoxicity, against autologous tumour, in the majority of TIL preparations tested. Two other groups, who also studied TILs, found them to have only negligible cytotoxic activity against autologous tumour cells [444, 15].

Of those who did not use autologous tumour as the target, one group, who also studied the histological features of the nodes, found that a high level of cytotoxicity was associated with paracortical expansion and sinus histiocytosis which represent an increase in

the T cell population [71]. Germinal centre hyperplasia, however, was associated with low levels of cytotoxicity possibly due to antigenic sites being blocked by ineffective antibodies [183]. Several groups tested the cytotoxicity of peripheral blood, lymph node and tumour infiltrating lymphocytes on a cell line, K562, which is particularly sensitive to natural killer (NK) cells and is used as a method of detecting their presence and function. PBLs appear to have a greater cytotoxicity against this cell line than lymphocytes from other sources, in patients with breast cancer [119, 50] and other malignancies [443, 445, 296], suggesting that this might be the major site of NK cells. Cunningham-Rundles *et al* [91] found that some patients had a very high NK cell function among their LNLs and Blanchard *et al* [42] found NK activity could be induced in lymphocytes obtained from a malignant pleural effusion by culture in IL-2. The relevance of the K562 cytotoxicity assay and the function of natural killer cells are still not clear.

In view of the severe limitations of the chromium release assay, a more precise method of studying cytotoxic function is required. Flow cytometry may be helpful in this area as damaged cells could be identified using propidium iodide (PI) to stain the nuclei, avoiding the need for chromium uptake and so

allowing the greater use of autologous tumour cells.

CELL MIGRATION

The migration of lymphocytes towards tumour cells, to encircle them and form clumps, has been noted by several observers [342, 103, 115, 228, 132] and found to be greater among lymph node lymphocytes than among peripheral blood lymphocytes [103, 115] or tumour infiltrating lymphocytes [342]. This method is very subjective and therefore results are difficult to compare or interpret.

The above *in vitro* studies are very indirect and take no account of the functional subgroups within each lymphocyte population.

SURFACE MARKERS

CELL PHENOTYPING

With greater understanding of the function of cells within the immune system, analysis of the phenotypic composition and activation, of a lymphocyte population

allows a clearer interpretation of its immune status. Using rosetting techniques, three groups compared lymphocytes from the axillary lymph nodes and peripheral blood in the same patients [116, 182, 171], and found a greater B cell population in the nodes while Vose & Moore [444] found the T and B cell populations in the tumour and the peripheral blood to be similar.

The development of monoclonal antibodies to the phenotypic markers on the cell surface membrane has greatly improved the accuracy of phenotyping and the use of flow cytometry allows dual immunofluorescent staining of both the phenotypic and activation markers on the same cell.

Three groups have used flow cytometry to compare lymphocytes from different sources within the same patient with breast carcinoma [295, 289, 272,].

The first of these studied only the phenotypic markers on peripheral blood and lymph node lymphocytes and found proportionally fewer T cells and fewer CD8+ T cells in the nodes, along with an increased CD4+/CD8+ ratio. This is equivalent to an increase in the proportion of B cells and CD4+ T cells in the nodes. Morton *et al* [289] also found an increased proportion of B lymphocytes and CD4+ helper T cells in the lymph nodes with CD4+ T cells predominating in stage I patients while B cells predominated in the nodes of patients with stage II

disease. They found HLA DR to be present on a higher proportion of T cells in the lymph nodes suggesting cell activation and antigenic stimulation and again this was greater in patients with stage II disease. As this study included no control subjects, they were unable to clarify whether these differences were due to lymphocyte source or the presence of malignant disease. They did not include analysis of the TILs or other activation markers such as the receptors for interleukin 2 (IL-2) and transferrin (Trf) nor did they seek evidence of a humoral immune response in the form of IgG production. Mantovani *et al* [272], using only single colour flow cytometry, compared the peripheral blood with invaded and uninvaded axillary lymph nodes. They found a greater B cell population in the lymph nodes regardless of metastatic involvement but found the CD4+ T cell population to be lowest in the uninvaded nodes while these were the nodes with the greatest HLA DR expression.

No studies have yet compared the lymphocytes infiltrating the tumour with those from the axillary lymph nodes and peripheral blood, within the same patients, with regard to phenotypic composition and cell activation.

PRESENT STUDY

In this study, the phenotypic proportions and activation status of lymphocytes from the primary tumour, axillary lymph nodes and peripheral blood were characterized in 22 patients with breast carcinoma, from whom all three samples were available. The aims of this comparison were to assess the loco-regional immune response at the time of presentation, to consider how it might be affected by conventional breast cancer therapy and to judge if the peripheral blood might be of use as a guide to the loco-regional immune status.

METHODS

Samples of primary tumour, axillary lymph nodes and peripheral blood were collected from 25 patients undergoing surgery for breast carcinoma which involved axillary node dissection. The cells were harvested from these samples as described previously and frozen in liquid nitrogen until they could be stained with monoclonal antibodies and analysed by flow cytometry. No TILs were obtained from 3 of the tumours and so they were excluded and the analysis performed on the remaining 22 patients.

As described in chapter 2, the cell suspensions were stained using the fluorescent antibodies listed in Table 1 which allowed the cells to be phenotyped as B lymphocytes, CD4+ helper T cells or CD8+ suppressor/cytotoxic T cells. These cells were also stained for HLA DR, the receptors for IL-2 and transferrin and, in the case of the B cells, surface IgG. As few B lymphocytes were found infiltrating the tumours the activation markers of these were not studied.

The phenotypes are expressed as a percentage of the total lymphocyte population while activation markers are given as a percentage of each phenotype bearing the marker.

In this study the phenotypic proportions and activation status of lymphocytes from the three tissue sources were compared to determine the major site of each phenotypic population and particularly the extent of the loco-regional response. The degree to which peripheral blood might be used as a guide to the status in the other tissues was assessed by correlating the phenotypic proportions and activation marker expression of the PBLs with those lymphocytes harvested from the regional lymph nodes and primary tumour.

RESULTS

PHENOTYPIC MARKERS

Lymph nodes yielded greater numbers of lymphocytes than the other sources with an average of 4 X 10^7 cells from each half lymph node compared with 1.5 X 10^7 cells from 10mls of peripheral blood. As the TILs were not separated from the tumour cells it was not possible to get fresh cell counts but it was clear from later analysis that, in most tumours, the lymphocyte yield was less than that from peripheral blood.

The major source of B lymphocytes was found to be the axillary lymph nodes where they accounted for 35% of the lymphocytes while the blood contained 13% and the tumour 12% ($p<0.001$) (Table 10) (Fig. 50). There was no correlation between the size of the B cell population at these different sites.

The axillary lymph nodes were also the major site of the CD4+ helper T cells which made up 49% of the LNLs, 39% of the PBLs and 30% of the TILs ($p<0.03$) (Fig. 51).

11% of LNLs and 25% of blood lymphocytes were CD8+ suppressor/cytotoxic T cells while these cells made up a much larger proportion of the TILs (41%) ($p<0.001$) (Fig. 52). Although these cells are clearly therefore the

PARAMETER	TILs (n = 20)	LNLs (n = 20)	PBLs (n = 20)	STUDENT'S t TEST
% T cells	63.8 ± 4.38	58.9 ± 3.12	60.6 ± 3.69	a. t=0.556 p=0.581 b. t=-0.399 p=0.694 c. t=0.923 p=0.362
% B cells	10.9 ± 1.76	34.8 ± 3.05	12.7 ± 1.29	a. t=-0.848 p=0.401 b. t=-7.39 p<0.0001 c. t=-6.599 p<0.0001
%CD4+ T cells	29.3 ± 2.91	48.4 ± 3.07	38.9 ± 3.13	a. t=-2.237 p=0.03 b. t=3.166 p=0.005 c. t=-4.509 p<0.0001
%CD8+ T cells	43.1 ± 3.39	11.4 ± 0.88	24.8 ± 1.90	a. t=4.820 p<0.0001 b. t=-7.116 p<0.0001 c. t=9.446 p<0.0001
CD4/CD8 RATIO	0.83 ± 0.15	5.5 ± 0.83	1.8 ± 0.23	a. t=-3.417 p=0.001 b. t=5.175 p<0.0001 c. t=-5.213 p<0.0001

Table 10 Comparing the phenotypic proportions among lymphocytes from the primary tumour, regional lymph nodes and peripheral blood of patients with breast cancer.
(a.) is the comparison of TILs and PBLs; (b.) the comparison of LNLs and PBLs and (c.) that of TILs and LNLs.

Fig. 50

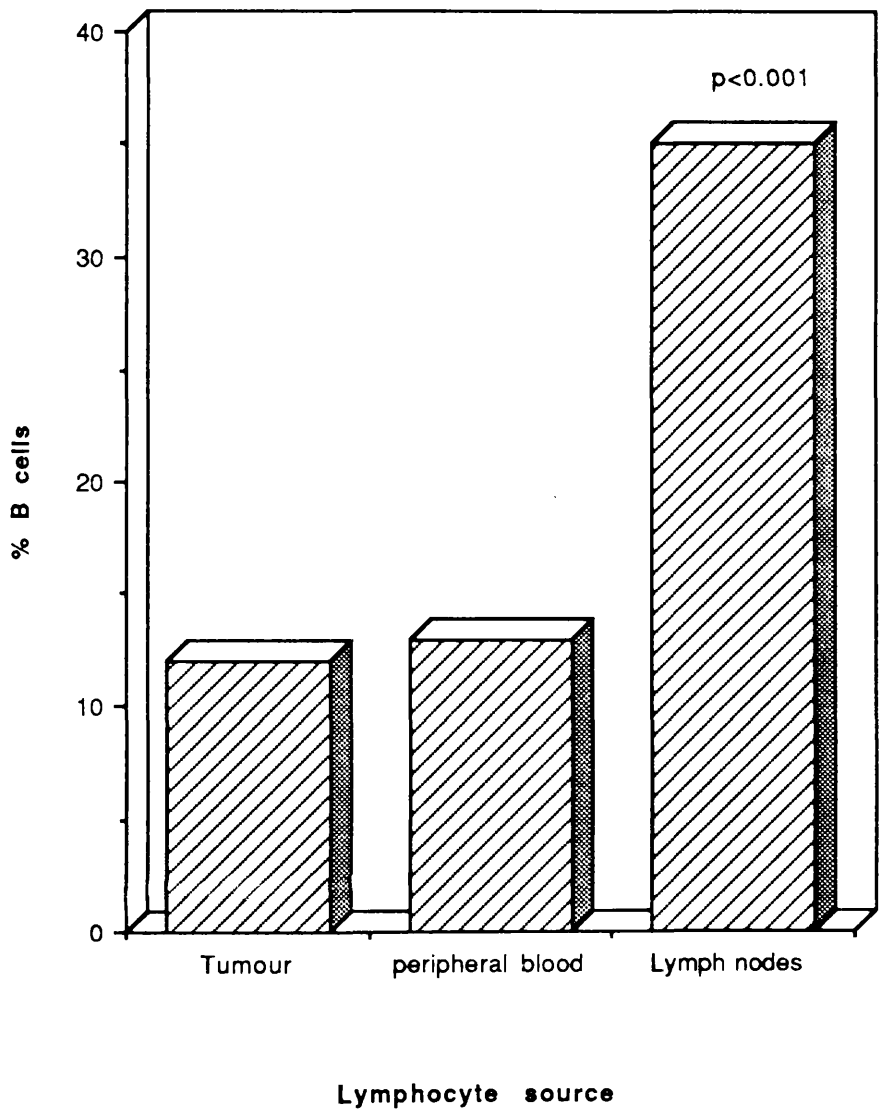

40
30
20
10
0
% B cells
p<0.001
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

Fig. 51

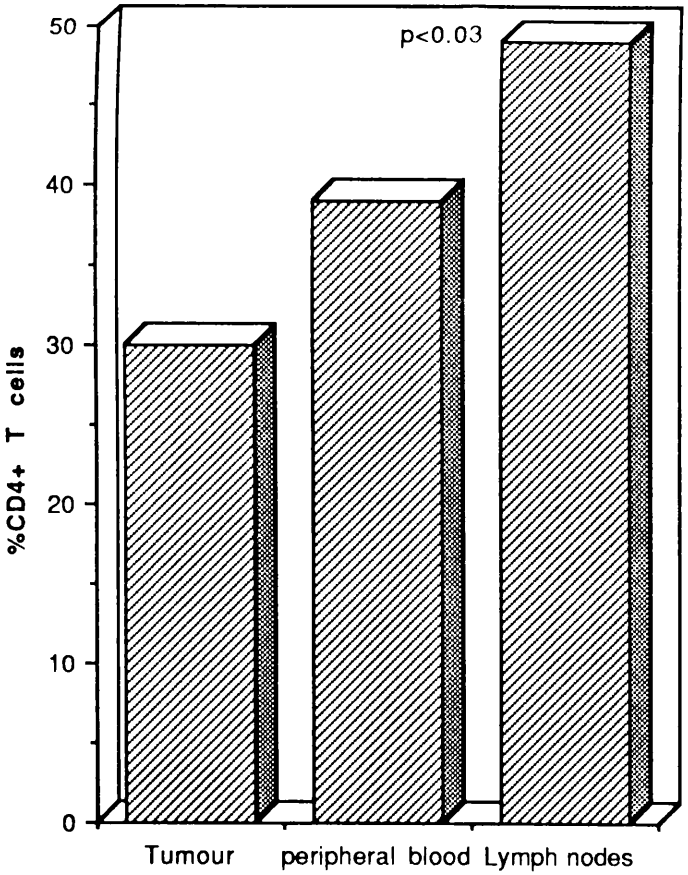
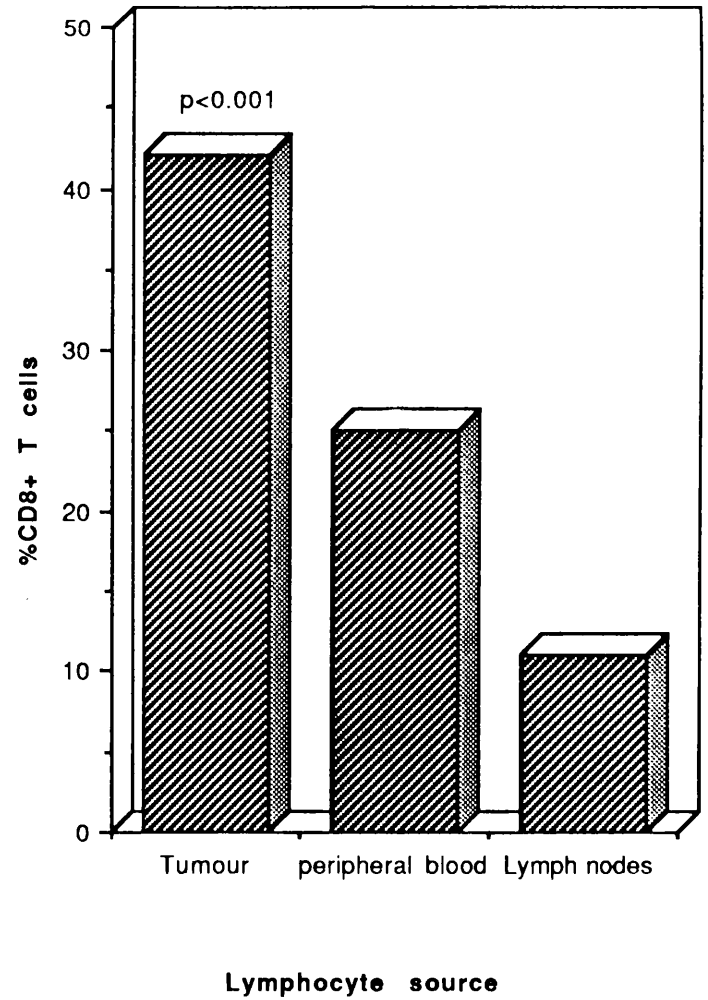

p<0.03
50
40
30
20
10
0
%CD4+ T cells
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

Fig. 52


p<0.001
50
40
30
20
10
0
%CD8+ T cells
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

largest component of the tumour infiltrate in many patients, the small quantity of infiltrate makes it difficult to harvest large numbers of CD8+ cells from this source.

Although the peripheral blood contained fewer CD4+ T cells it reflected the lymph node population with a strong positive correlation ($p<0.001$) (Table 11) (Fig. 53) while an inverse correlation was found, with regard to the CD8+ T cell population, between the peripheral blood and the regional lymph nodes ($p<0.03$) (Fig. 54). There was no correlation between tumour and blood for either T cell population.

Due to the above variations in the T cell subset proportions, the CD4+/CD8+ ratio was also found to vary markedly between the tissue sources, unlike the findings in normal subjects where there was little difference in the ratio between lymph node and peripheral blood lymphocytes (see chapter 2). The average subset ratio in the peripheral blood of breast cancer patients was 1.8 while that in the nodes was 5.5, reflecting the greater CD4+ population in the latter. The average ratio in the tumour samples was 0.8, due to the larger CD8+ T cell population at this site (Fig. 55). As the subset proportions correlated between lymph node and blood so did the ratio ($p<0.001$) (Fig. 56) although, as can be seen from the significance values, it is the CD4+

PARAMETER	CORRELATION OF PBLs and LNLs (n = 39)	CORRELATION PBLs and TILs (n = 20)	CORRELATION GRADIENT
% T cells	r = 0.148 p = 0.368	r = 0.222 p = 0.347	
% B cells	r = 0.285 p = 0.89	r = -0.001 p = 0.997	
% CD4+ T cells	r = 0.525 p = 0.001*	r = -0.030 p = 0.901	y=14.188+0.5247x R^2 = 0.276
% CD8+ T cells	r = 0.356 p = 0.027*	r = 0.160 p = 0.501	y=16.044+0.8389x R^2 = 0.127
CD4+/CD8+ ratio	r = 0.640 p < 0.0001*	r = 0.262 p = 0.265	y=0.836+0.1833x R^2 = 0.410

Table 11 Correlating the phenotypic proportions found in peripheral blood with those in the primary tumour and regional lymph nodes. The peripheral blood shows strong correlation with the T cell subsets in the nodes but not those infiltrating the tumour.

Fig. 53

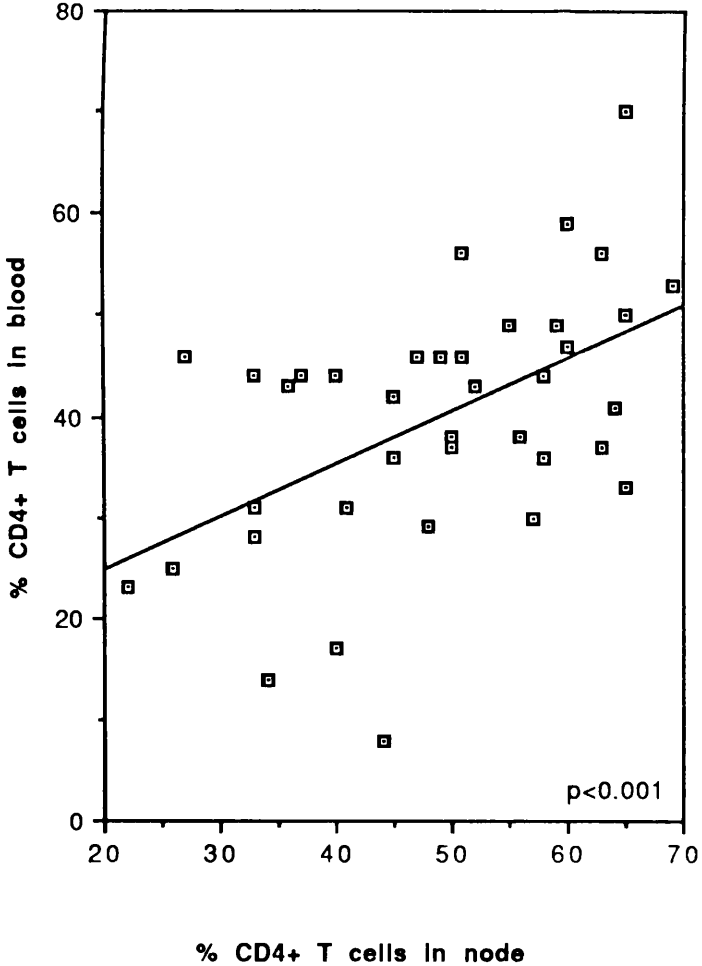

80
60
40
20
0
20
30
40
50
60
70
% CD4+ T cells in blood
% CD4+ T cells in node
p<0.001

Fig. 54

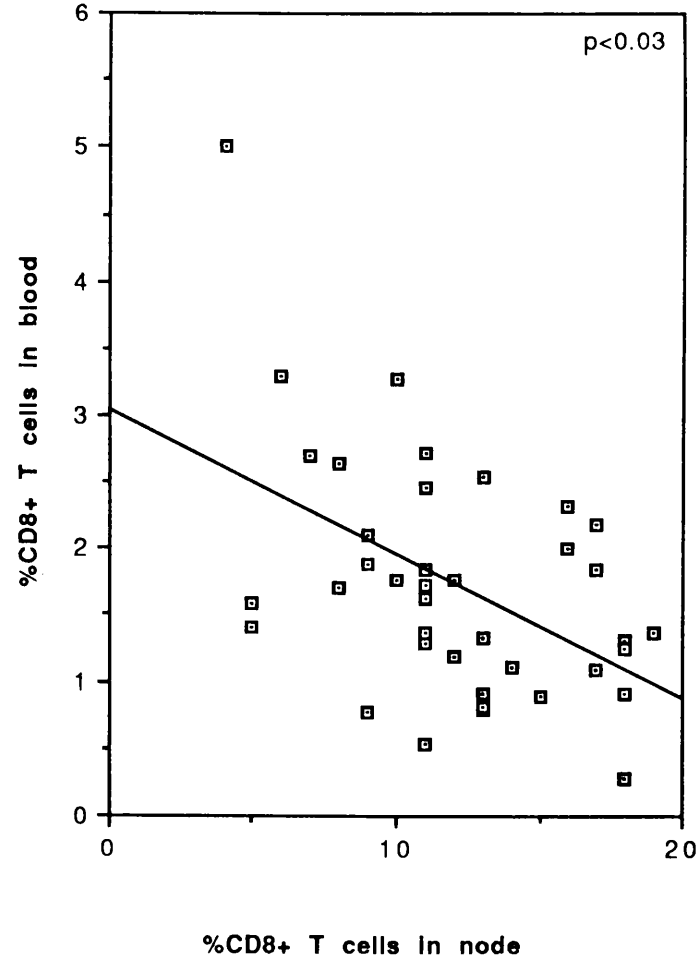

6
5
4
3
2
1
0
0
10
20
%CD8+ T cells in blood
%CD8+ T cells in node
p<0.03

Fig. 55

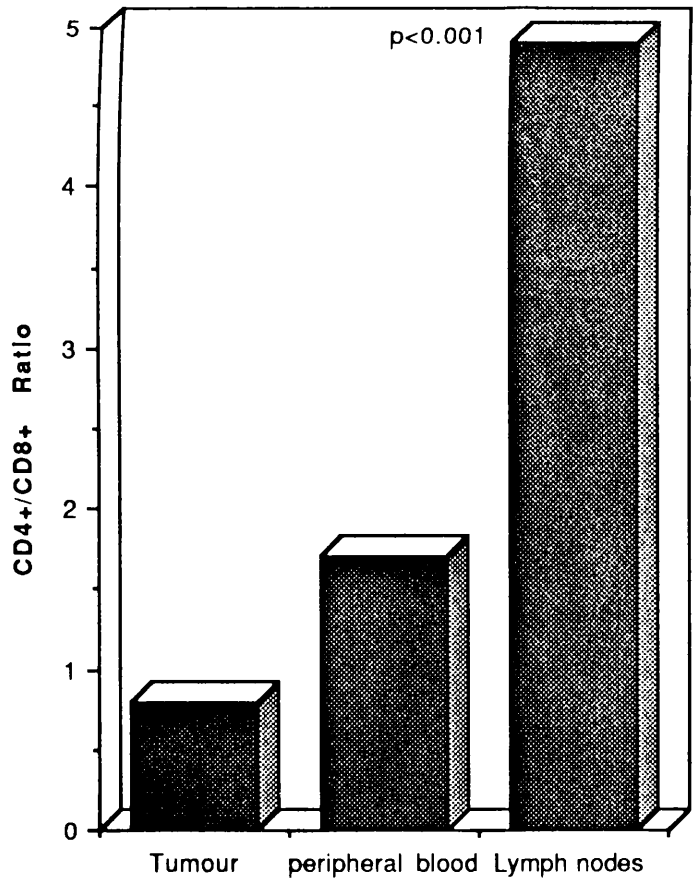
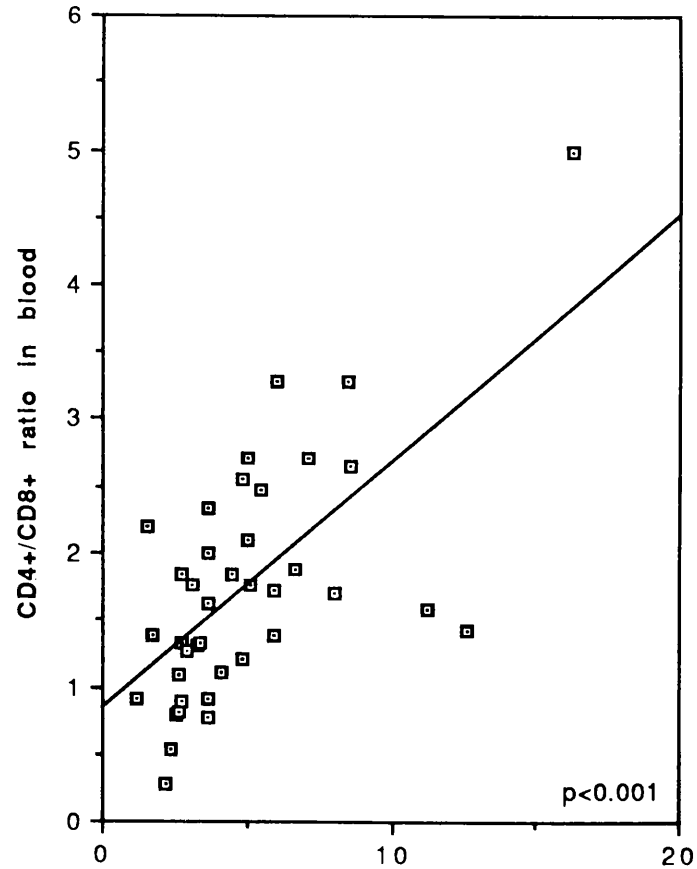

p<0.001
CD4+/CD8+ Ratio
0
1
2
3
4
5
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

Fig. 56


CD4+/CD8+ ratio in blood
0
1
2
3
4
5
6
0
10
20
p<0.001
CD4+/CD8+ ratio in nodes

population which appears to affect the ratio most strongly.

ACTIVATION MARKERS

HLA DR

The class II MHC antigen, was present on about half of the CD8+ T cells in both the tumour (53%) and the lymph nodes (50%) but on fewer of the circulating blood CD8+ T lymphocytes (37%) ($p<0.001$) (Fig. 57a).

The greatest proportion of CD4+ T cells bearing HLA DR was found to be within the tumour (46%) while 37% of the CD4+ cells in the lymph node and 20% of those in the blood also bore this activation marker (Table 12). The difference between tumour and lymph node was not significant but that between blood and the other sources was greater ($p<0.001$) (Fig. 57b).

There was no correlation between the expression of HLA DR on the CD8+ or CD4+ T cells in the blood with those from the regional nodes or within the tumour (Table 13).

Interleukin 2 receptor

The IL-2 receptor, was found on an average of 15% of all CD8+ T cells regardless of the tissue studied but as no significant correlation was seen between the cells in

Fig. 57a

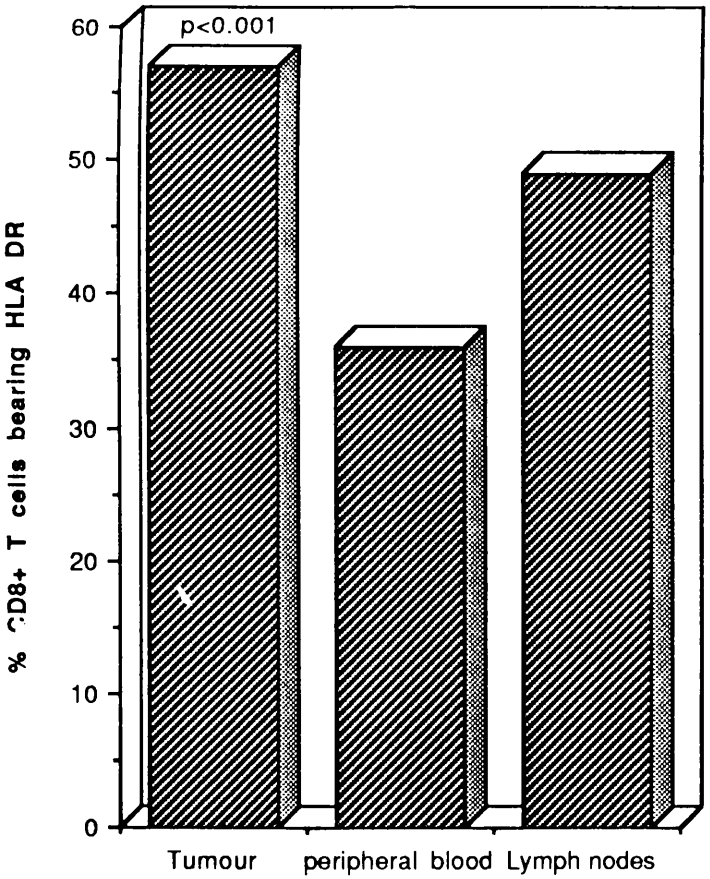
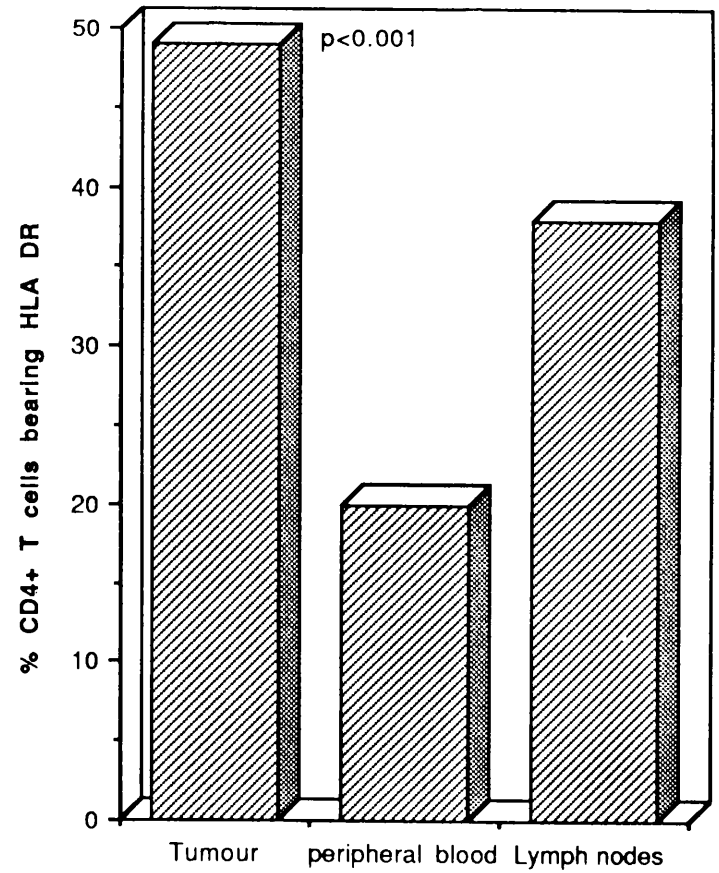


Fig. 57b



PARAMETER	TILs (n = 17)	LNLs (n = 17)	PBLs (n = 17)	STUDENT'S t TEST
%CD8+ HLA DR+	52.5 ± 4.47	50.1 ± 2.42	37.0 ± 2.23	a. t=2.597 p=0.02 b. t=4.327 p<0.0001 c. t=0.090 p=0.929
%CD8+ Tac+	14.3 ± 1.69	15.7 ± 1.47	16.1 ± 1.33	a. t=-0.971 p=0.347 b. t=-0.194 p=0.848 c. t=-0.984 p=0.341
%CD8+ TrfR+	39.8 ± 2.37	32.5 ± 2.34	38.0 ± 2.49	a. t=0.721 p=0.482 b. t=-2.933 p=0.008 c. t=2.193 p=0.044
%CD4+ HLA DR+	45.5 ± 4.31	37.5 ± 1.98	19.9 ± 1.60	a. t=7.738 p<0.0001 b. t=4.349 p=0.001 c. t=1.677 p=0.114
%CD4+ Tac+	27.2 ± 1.54	22.8 ± 1.45	33.1 ± 2.02	a. t=-3.589 p=0.003 b. t=-5.192 p<0.0001 c. t=2.563 p=0.023
%CD4+ TrfR+	47.0 ± 5.02	28.7 ± 2.83	34.3 ± 2.77	a. t=2.466 p=0.027 b. t=-3.060 p=0.008 c. t=3.166 p=0.007
%B high HLA DR		30.9 ± 3.23	10.3 ± 2.10	b. t=9.027 p<0.0001
%B Tac+		16.2 ± 1.17	22.9 ± 2.49	b. t=-3.286 p=0.004
%B TrfR+		42.5 ± 2.58	49.1 ± 2.74	b. t=-2.448 p=0.023
%B IgG+		33.3 ± 4.19	19.4 ± 2.02	b. t=3.753 p=0.001

Table 12 Comparing the activation status of lymphocytes from the primary tumour, regional lymph nodes and peripheral blood of patients with breast cancer.
(a.) is the comparison of TILs with PBLs; (b.) that of LNLs with PBLs and (c.) that of TILs with LNLs.

PARAMETER	CORRELATION OF PBLs and LNLs (n = 39)	CORRELATION PBLs and TILs (n = 17)	CORRELATION GRADIENT
CD8+ T cells bearing HLA DR	r = 0.233 p = 0.117	r = -0.313 p = 0.238	
CD8+ T cells bearing Tac	r = 0.219 p = 0.126	r = 0.172 p = 0.524	
CD8+ T cells bearing TrfR	r = 0.587 p < 0.0001*	r = -0.399 p = 0.126	y=18.934+0.5623x R^2 = 0.345
CD4+ T cells bearing HLA DR	r = 0.309 p = 0.064	r = -0.152 p = 0.589	
CD4+ T cells bearing Tac	r = 0.244 p = 0.107	r = 0.373 p = 0.171	
CD4+ T cells bearing TrfR	r = 713 p < 0.0001*	r = -0.019 p = 0.947	y=15.110+0.6763x R^2 = 0.509
B cells with high HLA DR	r = 0.700 p < 0.0001*		y=-1.439+0.4122x R^2 = 0.490
B cells bearing Tac	r = 0.560 p < 0.0001*		y=6.484+0.984x R^2 = 0.314
B cells bearing TrfR	r = 0.500 p = 0.001*		y=25.782+0.5332x R^2 = 0.250
B cells bearing surface IgG	r = 0.670 p < 0.0001*		y=6.663+0.3625x R^2 =0.449

Table 13 Correlation of the activation marker expression on blood lymphocytes with that on lymphocytes from the regional nodes and primary tumour. There is strong correlation of TrfR expression and all the B cell markers but not the T cell activation markers.

the tumour, node and blood, individual patients appeared to have this receptor on different proportions of CD8+ cells in the different tissues.

This receptor was found on a greater proportion of the CD4+ T cells in the tumour (27%) than in the lymph nodes (23%) but the greatest expression of this marker was on the circulating CD4+ T cells (33%) ($p<0.001$).

It was again noted that this receptor was carried by almost twice the number of CD4+ as CD8+ T cells regardless of the lymphocyte source ($p<0.001$) (Fig. 58).

Transferrin receptor

As seen in the previous chapter, there was no significant difference between the number of lymphocytes, of all phenotypes, bearing the transferrin receptor in the nodes and blood of breast cancer patients and of normal controls.

There were, however, more cells carrying this receptor among the CD4+ T cells infiltrating the tumour (47%) than from lymph node (29%) or blood (34%) ($p<0.05$) (Fig. 59).

The proportion of lymph node T cells, of either phenotype, bearing this receptor was clearly reflected in the peripheral blood ($p<0.001$) (Figs. 60a & 60b).

No significant difference was found in the activation

Fig. 58

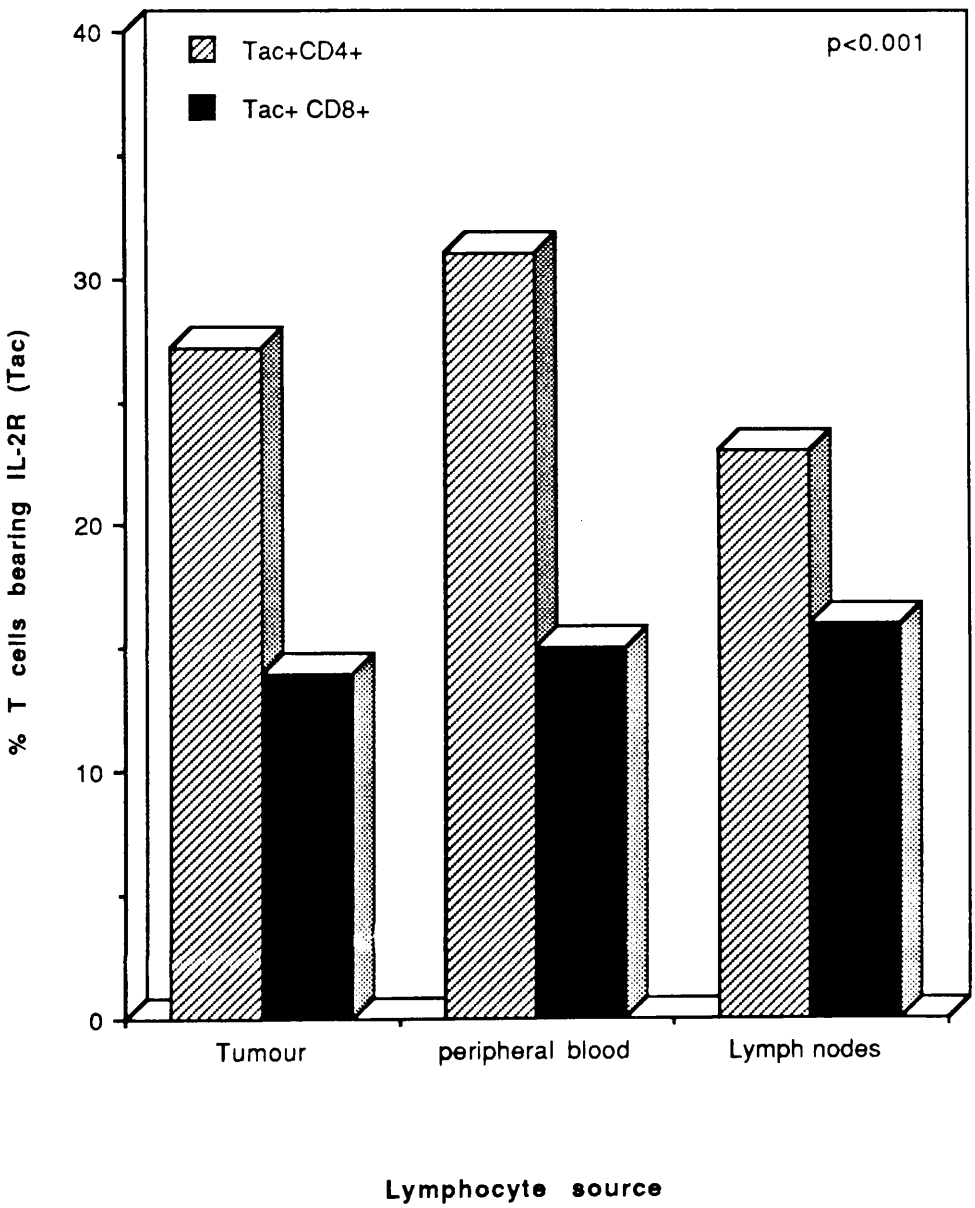

Tac+CD4+
Tac+ CD8+
p<0.001
40
30
20
10
0
% T cells bearing IL-2R (Tac)
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

Fig. 59

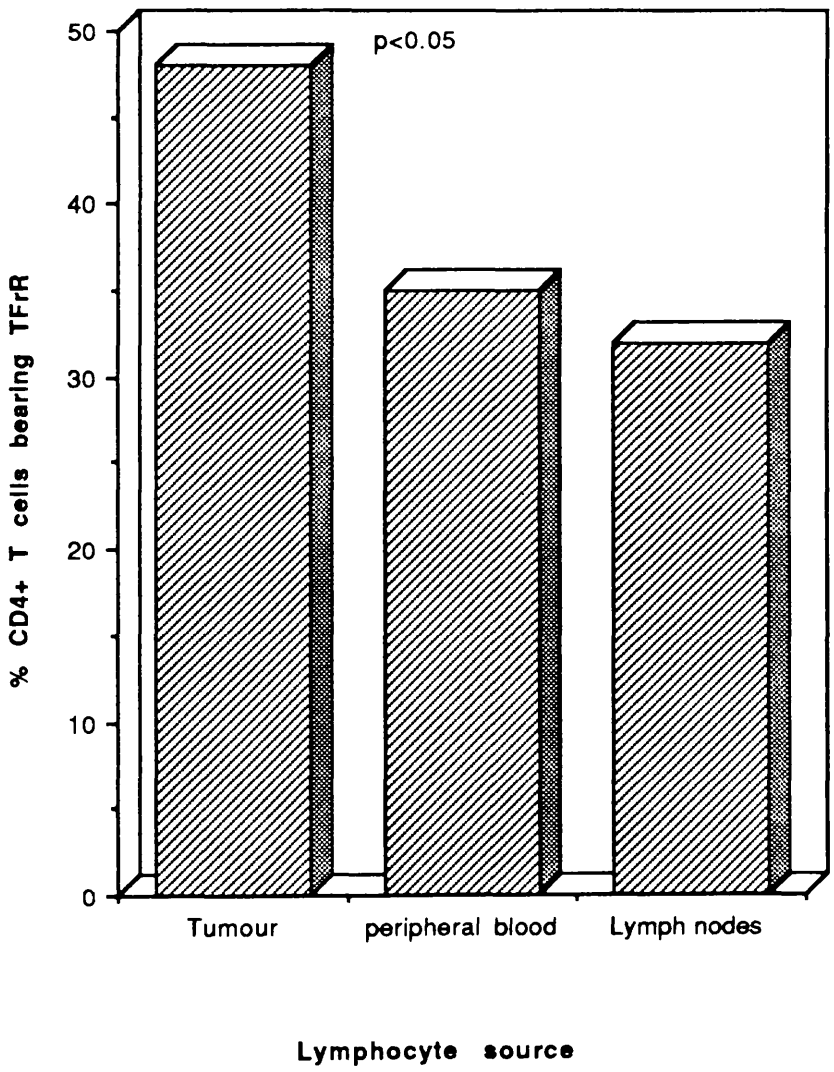

p<0.05
50
40
30
20
10
0
% CD4+ T cells bearing TFrR
Tumour
peripheral blood
Lymph nodes
Lymphocyte source

Fig. 60a

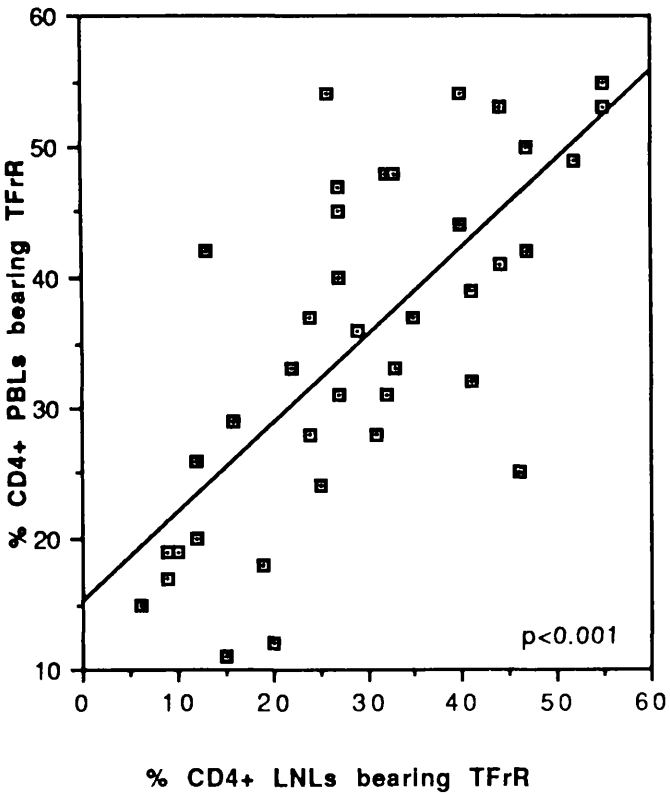
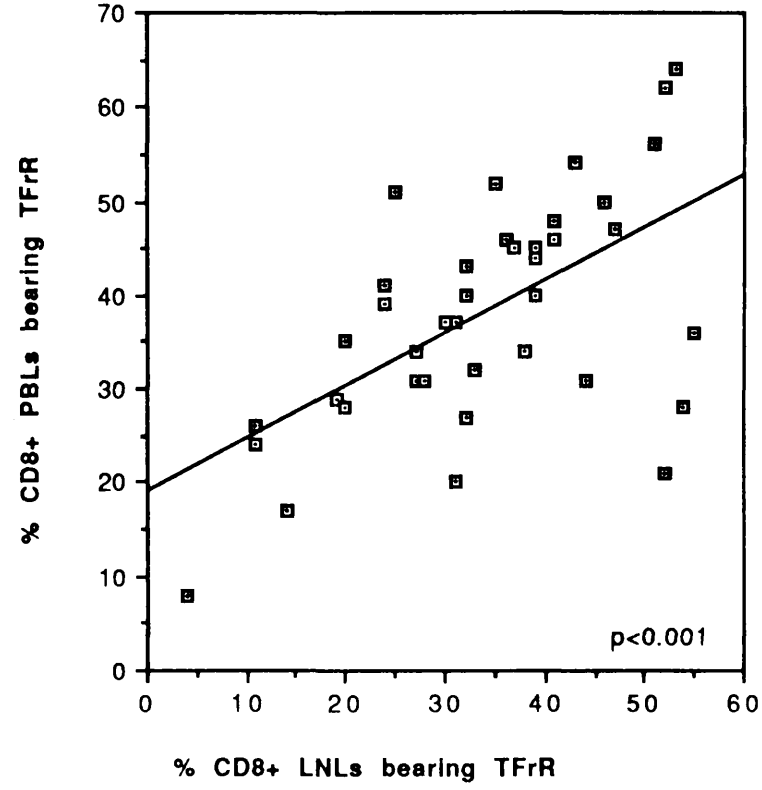


Fig. 60b



marker expression of the lymph node or peripheral blood B cells from patients with breast cancer or from normal controls but a strong correlation was noted between the two sources within individual patients ($p<0.001$) (Figs. 61a, 61b & 61c).

Immunoglobulin G

This was found on the membrane of 33% of the lymph node B lymphocytes as compared with 19% of the peripheral blood B lymphocytes ($P<0.001$) (Fig. 62). A broad range was seen in both sources, with the IgG population in the nodes varying from as little as 3% to as much as 70%, and the relationship between the two sources showing a strong correlation ($p<0.001$) (Fig. 63).

Fig. 61a

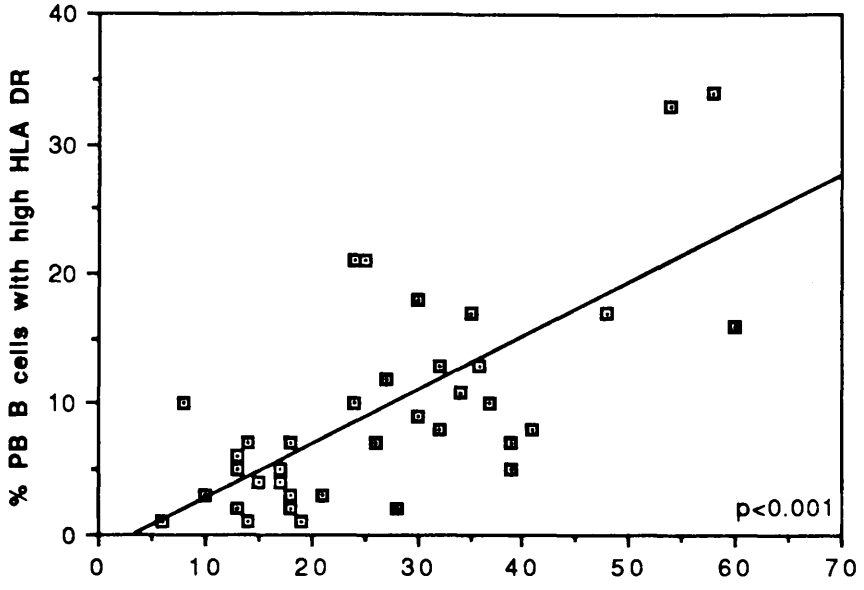


Fig. 61b

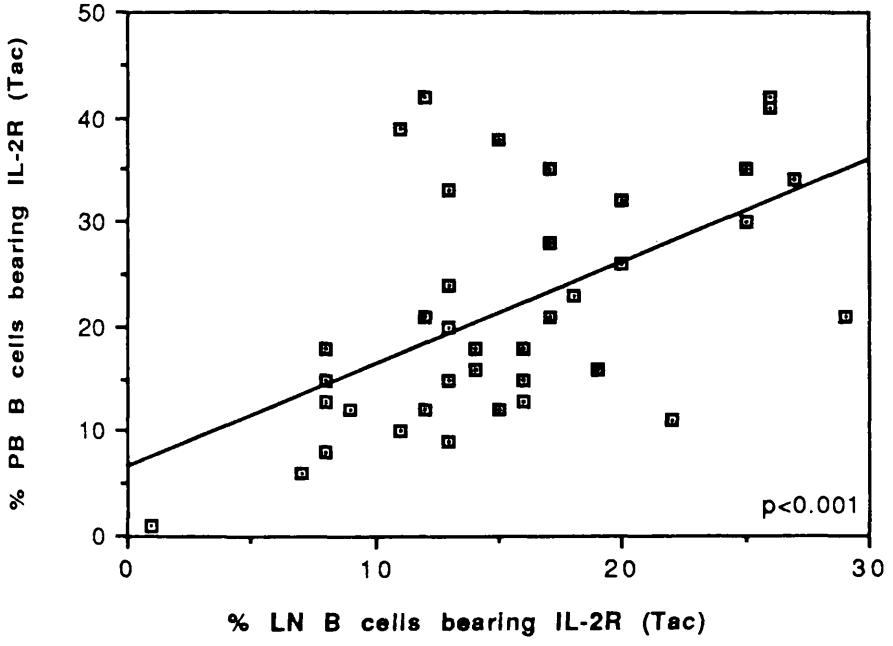


Fig. 61c

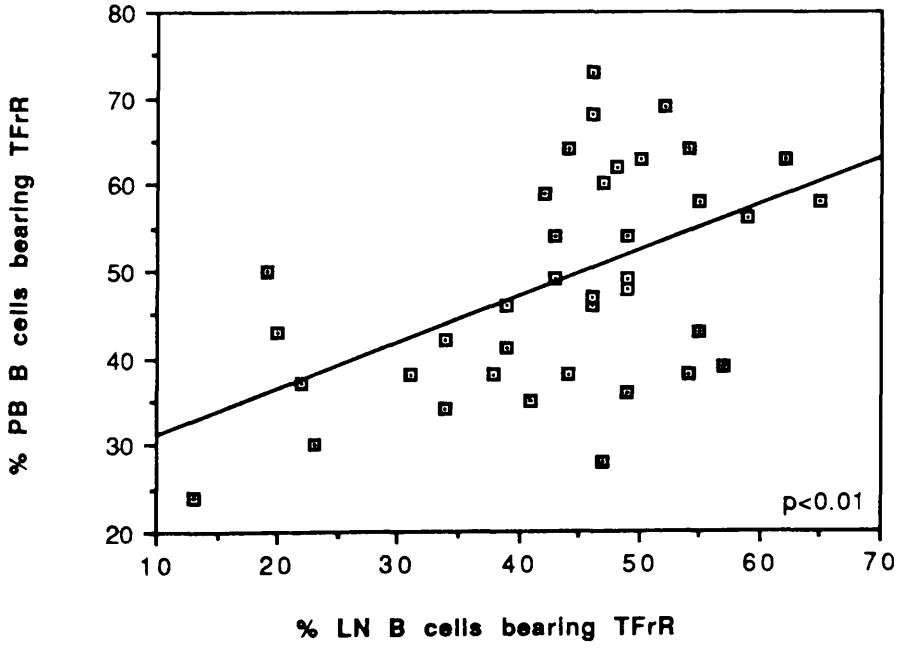


Fig. 62

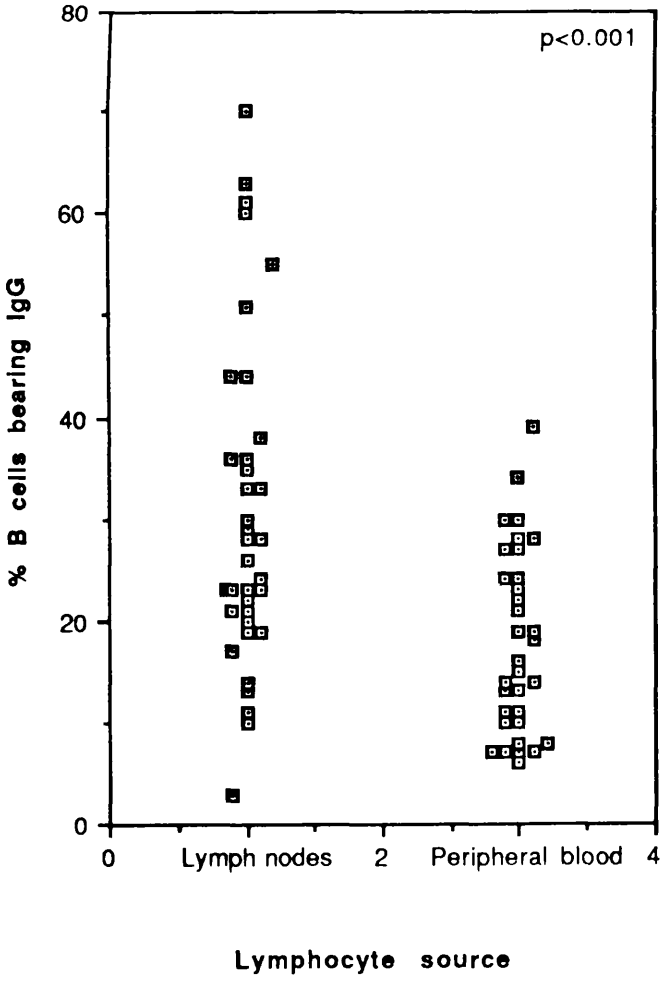
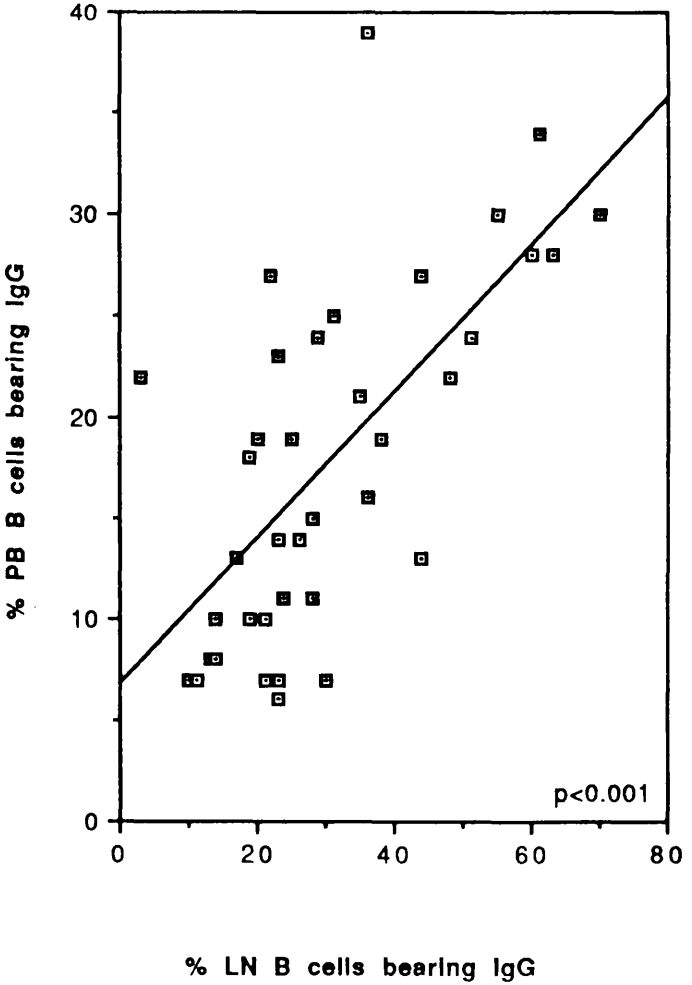

p<0.001
% B cells bearing IgG
80
60
40
20
0
0
Lymph nodes
2
Peripheral blood
4
Lymphocyte source

Fig. 63


% PB B cells bearing IgG
40
30
20
10
0
0
20
40
60
80
p<0.001
% LN B cells bearing IgG

DISCUSSION

PHENOTYPIC MARKERS

From these results it can be seen that there is great variation in the phenotypic proportions between the three sources. The axillary lymph nodes appear to be the major site of the B lymphocyte population which is in agreement with previous studies which have compared axillary lymph node and peripheral blood lymphocytes, either by using surface immunoglobulin and rosetting techniques [182, 118] or monoclonal antibodies and flow cytometry [289, 295] to identify the B cells.
As with many previous studies (see chapter 2) we found few B lymphocytes within the primary tumours.
Studying the T cell subsets, we found that the regional lymph nodes were also the main site of the CD4+ T cells, with a smaller proportion of CD8+ T cells than peripheral blood. This is similar to the findings of Morton *et al* [289], Nakajima *et al* [295] and Mantovani *et al* [272] who compared the PBLs and LNLs using flow cytometry. While this last group found a smaller CD8+ T cell population within the regional nodes, they found no increase in the CD4+ T cell population and indeed a fall in this cell type in the uninvaded nodes.

Although other groups have found no relationship between the phenotypic proportions of the PBLs and LNLs [318, 456], we did find correlation between the size of the CD4+ T cell population in the axillary nodes and peripheral blood and, because of this, the CD4+/CD8+ ratio also correlated strongly. The size of the CD4+ T cell population and the CD4+/CD8+ ratio of the PBLs might therefore give some indication of those patients with an increased proportion of CD4+ helper T cells in their axillary nodes.

Controversy exists over the T cell subset proportions among the tumour infiltrating lymphocytes (see chapter 2) but in this study the CD8+ T cells were found to predominate. The cell yield from the tumours is not as high as that obtained from lymph node or blood and this may not therefore be the greatest quantitative source of these cells.

ACTIVATION MARKERS

HLA DR

In chapter 2 it was shown that this activation marker was present on more than twice as many T lymphocytes in the patients with breast cancer as in the controls. In this study, comparing the different lymphocyte sources,

this marker is seen to be on a large proportion of the CD8+ T cells infiltrating the tumour and present in the axillary nodes. In the TILs this proportion increases to include almost all of the CD8+ T cells in poorly differentiated tumours which express the class I MHC complex and contain a strong lymphocytic infiltrate. In the nodes the expression of HLA DR on the CD8+ T cells is related to tumour stage and thus to the direct contact of these cells with metastatic tumour deposits. Both of these findings reinforce the association of this activation marker with antigen presentation.

As in the study by Morton *et al* [289], we found many more lymphocytes, of all phenotypes, carrying HLA DR in the axillary lymph nodes than in the peripheral blood and this presence of so many HLA DR bearing lymphocytes in the regional lymph nodes suggests that the activation and antigen presentation seen within the tumour also involves the axillary lymph nodes particularly, though not exclusively, if the tumour has spread to these nodes.

Interleukin 2 receptor

The IL-2 receptor was found on virtually the same proportion of CD8+ cells from each source but was on more CD4+ T cells in the peripheral blood with much greater variation between the different sites. Neither

Morton *et al* [289] nor Mantovani *et al* [272] included this receptor in their studies.

As this receptor is present on almost twice as many CD4+ T cells as CD8+ T cells, regardless of the source, it may be that either the CD4+ T cells are more activated than the CD8+ T cells or that there is some functional difference between the two with regard to binding and stimulation by IL-2. This lymphokine is being considered as a possible therapeutic agent and this difference therefore requires further investigation.

Transferrin receptor

The Trf receptor was found to be present on slightly more lymphocytes in the nodes and blood of breast cancer patients than of normal controls but this was not statistically significant. The highest level of expression of this receptor was among the tumour infiltrating CD4+ T cells suggesting there was greater expansion among this population. While there was no great difference between cancer patients and control subjects, a very strong correlation was seen between the transferrin receptor expression on lymphocytes, of each phenotype, from the axillary nodes and peripheral blood of individual patients suggesting that the variation between patients may be due to constitutional differences rather than the presence of malignant

disease. This marker has not been included in any other studies comparing lymphocyte sources within patients with breast cancer.

Immunoglogulin G

It can be seen from the results presented in chapter 2 that breast cancer patients have more mature IgG bearing B lymphocytes and, in this study, the axillary lymph nodes were found to be the major source of these lymphocytes. The proportion of peripheral blood B cells bearing surface IgG was found to correlate strongly with that in the nodes and might therefore act as a useful guide to the presence of a regional humoral immune response.

It is notable from these findings that all the parameters on B cells correlate strongly between lymph node and blood. This finding suggests that these cells circulate more freely between the tissues than the T cell populations.

CLINICAL IMPLICATIONS

At the present time, the conventional treatment for breast carcinoma involves surgical excision of the

primary tumour along with the axillary lymph nodes and this is often followed by adjuvant therapy which includes loco-regional radiotherapy to the breast, axilla or both. Clearly, any regional immune response is likely to be destroyed by this management and indeed studies of the circulating lymphocytes after radiotherapy show a loss of the CD4+ helper T cells [330, 331, 425, 213, 378, 305], which persists for many years after the initial disturbance [330, 360, 305], and a loss of responsiveness to mitogens [397, 424, 451, 63], which was found, by one group [451] to be prognostic of long term outcome. Radiotherapy has also been shown to reduce both the B cell population [449] and immunoglobulin secretion which are based in the axillary nodes [397, 450].

If cytotoxic chemotherapy is also used then the patients suffer from generalised immunosuppression which again decreases the immunoglobulin secretion of B cells [472] and affects the CD4+ helper T cells which do not recover easily [401, 266, 332, 44, 378, 227]. However, recent concensus analysis, of many systemic adjuvant therapy trials, does suggest an improvement in survival for premenopausal women with stage II breast cancer treated with chemotherapy and for postmenopausal women treated with Tamoxifen [110]. Accurate staging of the disease is therefore required for the logical planning of adjuvant

therapy.

Clinical staging is not very accurate, as has been borne out by several studies [95, 133], although the findings of the NSABP trial would also suggest that it is unimportant whether microscopically involved nodes are removed prophylactically or only upon clinical presentation. Indeed 60% of these patients will not progress to develop clinical disease in the axilla [133]. This does not help in planning the management of individual patients and various approaches have therefore been tried to improve staging while avoiding an axillary clearance.

Scintigraphy, using injections of colloid or monoclonal antibodies, has been tried by several groups [2, 41, 333, 151], but while some groups remain hopeful about the eventual application of this technique [221, 217], the results are difficult to interpret and subsequent axillary dissection has shown this method to be unreliable [422].

Other groups considered the possibility of sampling a few lymph nodes lying low in the axilla [134, 144, 230, 391] or even just at the pectoral margin, within the mastectomy specimen [144, 391]. While some felt this reduced dissection was sufficient [144, 391], others maintained that a greater clearance was necessary [134] or that at least a minimum of 4 axillary lymph nodes should

be retrieved to achieve reliable staging [230].
As this often requires considerable dissection of the lower axilla and it has been suggested that this is the site of the most activated nodes [130], it is likely that even limited excision sampling of the axillary lymph nodes will result in damage to any regionally based immune response.
A recent article has suggested that a thorough dissection of the axilla is mandatory to allow accurate staging of breast carcinoma and the selection of the appropriate adjuvant therapy [125]. It is also implied that axillary clearance itself makes a major therapeutic contribution to the outcome but while the former argument may be valid there is little evidence to support the latter.
The major indication for axillary lymph node excision sampling is to stage the disease as this remains the most important single prognostic indicator [48] and criterion for adjuvant therapy.
Although lymph node stage remains the most important prognostic indicator [48], it is by no means accurate with 30% of patients with stage I breast cancer still dying from their malignant disease.
As features of the tumour itself, such as oestrogen receptor status, c-erbB-2 (neu) oncoprotein expression and the levels of urokinase-type plasminogen activator

(u-PA), are identified as useful prognostic indicators the role of lymph node staging may diminish, allowing the axilla to be considered from a therapeutic rather than diagnostic point of view.

In this study, the peripheral blood was found to give some indication of alterations in the phenotypic proportions and activation marker expression of lymphocytes within the axillary lymph nodes of breast cancer patients. This was particularly so with regard to the relative proportions of CD4+ helper T cells and CD8+ suppressor/cytotoxic T cells and the humoral response of the B cells. Including this analysis in the preoperative assessment might allow the selection of patients, deemed to be mounting a response, to be considered for conservation of the axillary lymph nodes and the use of non-immunosuppressive adjuvant therapy rather than the more destructive modalities such as radiotherapy or chemotherapy.

Several studies suggest that while the progesterone receptor blocking agent, megesterol acetate, does appear to cause some immunosuppression [369, 324] the oestrogen blocking agents do not [214, 324, 440] and one group even found them to cause some stimulation of the immune system [301].

Enhancement of any immune response may also be possible using biological response modifiers. Although there are

other biologically active substances which can be considered for anti-tumour therapy [145], such as alpha interferon [172, 51, 293, 303], and tumour necrosis factor [226, 43, 379], IL-2 has shown the greatest promise in the treatment of solid tumours [314, 313]. IL-2, which was originally called T cell growth factor, was first isolated from the supernatant of PHA stimulated lymphocyte cultures [285].

Since recombinant IL-2 first became available [411] its clinical potential has been studied extensively by Rosenberg and his group who first demonstrated that peripheral blood lymphocytes, from all patients, could be stimulated *in vitro* to produce lymphokine activated killer (LAK) cells [179] which were cytotoxic to fresh tumour cells. The anti-tumour potential of IL-2 has been demonstrated as a single agent [256, 257, 11], in combination with LAK cells [292, 337], or more recently in combination with IL-2 stimulated tumour infiltrating lymphocytes (TILs) [427, 356].

These various forms of IL-2 therapy have been tried in the treatment of many tumours [314] including malignant melanoma [355], renal cell carcinoma [355], bladder carcinoma [334], lymphoma [455] and tumours of the head and neck [404] but this approach has, as yet, been tried in only a few patients with breast cancer who have been included in mixed trials [61, 225, 32, 219, 229, 363].

The initial response rates have not always been maintained as more centres have tried to use this therapy [455, 142, 109], but there are still reports of lasting remissions.

The present use of IL-2 is as a growth factor, allowing the expansion of TILs which are harvested from the patient's tumour, cultured in IL-2 and then returned to the patient as a systemic therapy for metastatic disease [354, 389, 220]. It is thought that IL-2 works by expanding and activating the cytotoxic T cells from the tumour which, upon return to the patient, destroy the metastases by direct cell killing. However, where the phenotype of the TILs has been studied, it has frequently been found that while those harvested from the patient are predominantly CD8+ cytotoxic T cells, those being infused back into the patient are mostly CD4+ helper T cells. Thus the greatest expansion appears to be in the CD4+ helper T cell subset with a concomitant decline in the proportion of CD8+ cytotoxic T cells causing the helper/cytotoxic ratio to reverse after two or three weeks in IL-2 stimulated culture [22, 185, 294, 242, 276, 386]. From our results it would appear that more CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells carry the receptor for this lymphokine and this may be the reason for their preferential expansion. There are also some studies, in

animals and humans, which suggest that CD4+ T cells cells play a greater role in the rejection of allografted kidneys than the CD8+ T cells [78, 49, 166]. It is possible, therfore, that the responses to TIL therapy may have been mediated by the CD4+ helper T cells rather than the CD8+ suppressor/cytotoxic T cells. If this is the case, culturing and expanding the large CD4+ T cell population from the regional nodes may be more effective. The huge cost of culturing individual cell therapies for patients makes the study of this approach very difficult but the regional instillation of IL-2 could be considered. Some mechanism such as microsphere delivery could be used, to reduce spillage into the systemic circulation and avoid some of the extreme side effects experienced when intravenous administration has been used [257]. Other biological response modifiers, such as alpha and gamma interferon, should also be considered [145, 246]. These have been found to induce the expression of class I and class II MHC complexes on tumour cell lines [157, 55, 210] and might therefore improve antigen recognition, by the immune system, of those tumours with poor expression of these complexes.

CONCLUSIONS

From these results it would appear that the lymphocytes involved in the host anti-tumour immune response, in patients with breast carcinoma, are sited within the primary tumour or axillary nodes.

The axillary nodes are the main site of the IgG bearing B lymphocytes in patients who appear to be mounting a humoral immune response to their tumours. The antibodies secreted by these cells require to be immortalized and studied to assess their specificity and function.

These nodes are also the major site of the CD4+ T cell population in patients with a high CD4+/CD8+ ratio although those in the tumour appear to be more actively dividing. These cells require to be analysed with regard to lymphokine secretion to assess their functional contribution, as helper T cells, to the anti-tumour immune response.

The CD8+ T cells make up the greater proportion of cells infiltrating the tumours although the regional nodes are the greater quantitative source. The strong correlation between the proportion of CD8+ TILs bearing HLA DR and both tumour grade and class I MHC antigen expression suggests that the primary lesion is the most relevant source of these cells. The importance of direct contact

between CD8+ T cells and tumour cells is also supported by the correlation seen between the expression of HLA DR on these cells in the axillary nodes and the presence of nodal metastases.

Cytotoxicty assays are required to assess the functional capacity of these cells but a more sensitive and accurate method than the currently used chromium release assay is needed. Flow cytometry may also have an application in this area.

The distribution of each phenotypic population is in keeping with our knowledge of their function. As both the B lymphocytes and CD4+ helper T lymphocytes have a primarily secretory function, the one producing immunoglobulin and the other lymphokines, they do not require to be situated within the tumour but can perhaps function better within the regional lymph nodes where they can communicate with other components of the immune system not present in the tumour. The CD8+ suppressor/cytotoxic T cells, however, function by recognizing antigenic structures on the surface of the tumour cell in conjunction with the class I MHC complex. This is followed by direct cell to cell killing. They must therefore be based within the tumour to allow access to the antigenic tumour cells. These cells appear to be important in the initiation of an immune reaction as well as representing the functional

cytotoxic component of the cellular immune response. While some parameters of phenotypic proportions and cell activation are reflected among the circulating lymphocytes, this is generally to a lesser degree suggesting that the main response is still loco-regional at the time of clinical presentation.

The components of this loco-regional immune response are therefore likely to be destroyed by conventional surgery or radiotherapy and, if chemotherapy is used, the entire immune system is depressed. While these modalities may be indicated in many patients, those with evidence of a regionally based response should perhaps be selected for conservation of the axillary nodes and even immunotherapy such as regional administration of IL-2. Analysis of the phenotypic proportions, and their surface activation markers, within peripheral blood samples may be useful as an indicator of the regional immune status and would allow the preoperative selection of patients to be considered for axillary lymph node conservation and immunotherapy.

CHAPTER 4: HUMAN MONOCLONAL ANTIBODIES TO BREAST CARCINOMA

INTRODUCTION

There are three major possibilities for immunotherapy by manipulation of the immune response of breast cancer patients.

1) The patient's own response may be enhanced *in vivo* using such lymphokines as interleukin 2 or by inducing tumour cell MHC complex expression with alpha or gamma interferon.
2) The cellular immune response may be harvested and augmented by *in vitro* culture and expansion before being returned to the patient.
3) The humoral response may be isolated in the form of a human monoclonal antibody which could be used in patients who are not mounting a response.

While the first approach has some potential, as discussed in chapter 3, the second is limited because of the logistical difficulties of developing a completely individualized therapy for each patient.
The advantages of monoclonal antibodies are that they

could be used in other patients and would also be useful in the research field to allow the isolation and characterization of tumour associated antigens.
The results presented in the previous chapters suggest that some patients are mounting a humoral immune response and, as the specificity of that response can only be studied when it has been isolated and immortalized in the form of a monoclonal antibody, we wished to look at the feasibility of such antibody production.

HISTORICAL REVIEW

MURINE MONOCLONAL ANTIBODIES

Since the first monoclonal antibodies were produced by Kohler and Milstein in 1975 [235], attempts have been made to apply them to the detection and treatment of cancer. Some antibodies of murine origin have been of use in the histological examination of tumours [152, 215, 306, 376, 299] and for the *in vitro* detection of bone marrow metastases [81, 158, 405, 114, 319, 9, 253]. However, while many localization studies have been carried out, attempting to diagnose [370, 75, 286, 168, 270, 237] or treat breast cancer [453, 218, 312, 162], their use *in vivo* is severely limited.

HUMAN ANTI-MOUSE RESPONSE

One of the major problems of murine monoclonal antibodies is that the recipient tends to raise an immune response against the foreign immunoglobulin which can result in an adverse reaction to the antibody if it is administered more than once [453, 312, 162]. At its most severe this reaction can cause serum sickness,

although this is rare, or a milder reaction may simply result in the destruction and excretion of the murine antibody before it is able to localize within the tumour [168]. The antibody and its conjugate, which may be a radionuclide, cytotoxic drug or toxin, form immune complexes with the patient's anti-mouse antibody and are trapped in the reticulo-endothelial system thereby greatly increasing the cytotoxic dose to the liver and spleen [218]. These immune complexes may also settle out in the kidney causing tubular damage (Fig. 64).

To avoid this problem several groups have tried to produce chimaeric antibodies which still have a murine variable region but a human constant region [288, 54, 408, 216, 377, 365, 310, 395, 174, 254] (Fig. 64). These appear to have had some success in increasing the circulation time [254] with only a few patients developing a detectable immune response to the mouse variable region. Some success was reported when a chimaeric antibody, to a lymphocyte phenotypic marker, was used to eliminate all the lymphoid cells in two patients with non-Hodgkins lymphoma leaving the haemopoetic stem cells intact [174] but there have been no further reports of this approach.

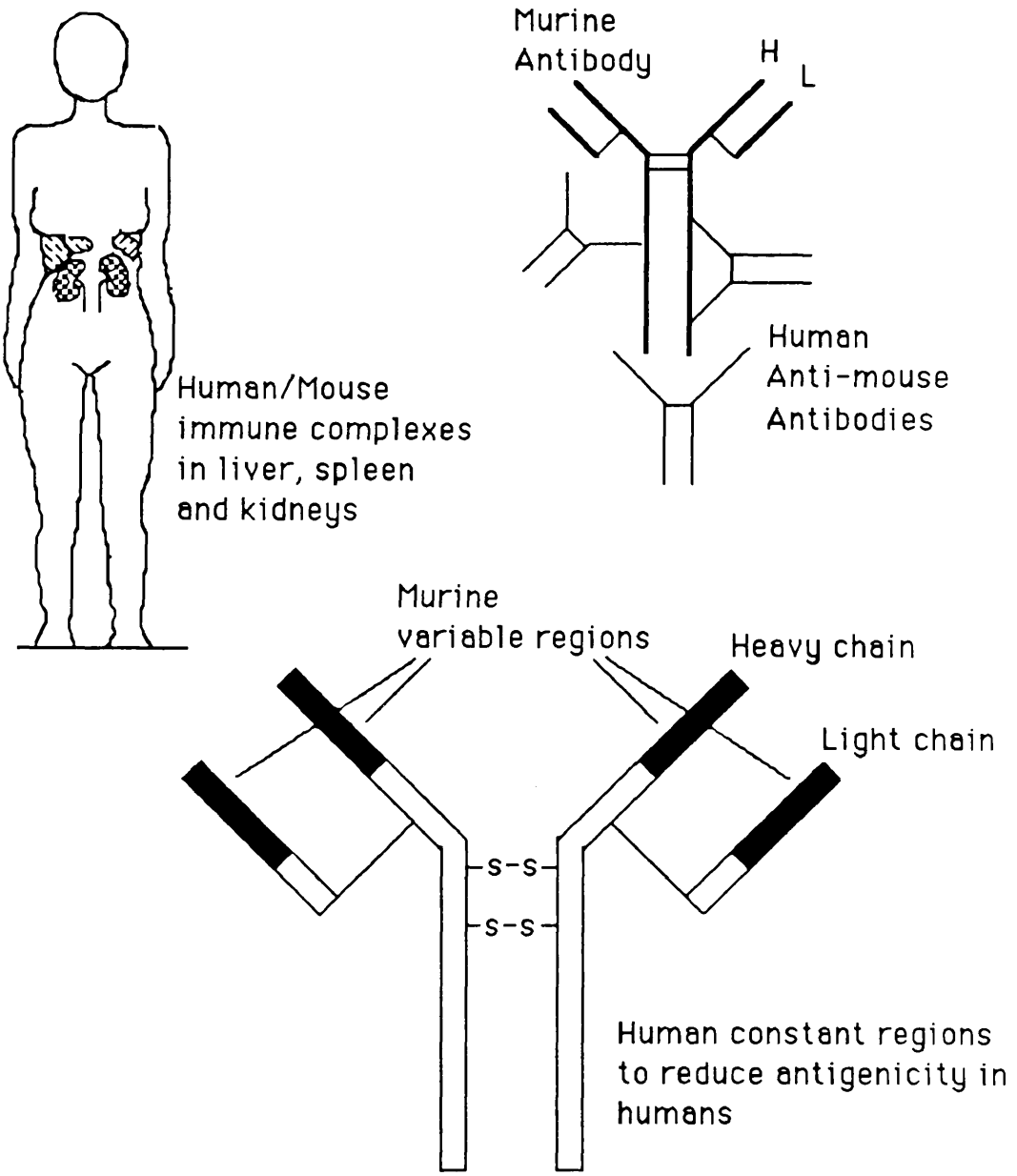


Fig. 64 Mouse monoclonal antibodies cannot be administered repeatedly because the recipient develops antibodies against the murine immunoglobulin and the resultant immune complexes cause damage to the kidneys and the reticulo-endothelial system. Humanising murine prolongs their circulation time.

LACK OF SPECIFICITY

Unfortunately the development of these chimaeric antibodies does not overcome the greatest problem of murine monoclonal antibodies which is poor anti-tumour specificity. *In vivo* administration of these antibodies leads to non-specific binding with normal tissues and poor tumour localization [218, 237].

As yet no clear tumour specific antigen has been isolated which could be used to produce a murine monoclonal antibody against breast cancer.

Some groups suggest that breast cancer is caused by a viral agent, similar to the mouse mammary tumour virus and that this could therefore be used as an antigen to raise monoclonal antibodies in mice [39, 275, 381, 5] but the commonest breast cancer antigens, to which murine antibodies react, are high molecular weight glycoproteins [421, 267], many of which appear to be components of the milk fat globule [413, 62, 190, 169]. These antigens have not been well characterized and, as they were initially isolated using murine monoclonal antibodies, their relevance to human breast tumours remains in doubt. If a patient raises an immune response against her tumour, it is because her immune system recognizes it as foreign and the response is therefore likely to be directed against tumour-specific

antigen whereas if a mouse is inoculated with a human tumour cell the overwhelming mouse anti-human immune response will predominate and make it almost impossible to detect any mouse anti-tumour reaction (Fig. 65). This problem is particularly illustrated by the difficulty of making murine monoclonal antibodies to the Rhesus D blood group antigen. The response is dominated by the reaction of the mice to the major ABO blood group antigens [56, 84, 59, 419, 66] and it is this difficulty which has given much of the impetus to the search for human monoclonal antibodies.

HUMAN MONOCLONAL ANTIBODIES

To address these problems, of repeated administration and tumour antigen specificity, human monoclonal antibodies are required. These would be of importance not just clinically, for diagnosis or therapy, but also scientifically allowing isolation and identification of tumour specific antigens. Production of human monoclonal antibodies was first attempted by Steinitz *et al* in 1977 [394] using Epstein Barr virus (EBV) to immortalize the stimulated lymphocytes removed from the patient. Although some human antibodies have been used to isolate breast cancer antigens [412, 154, 352, 206,

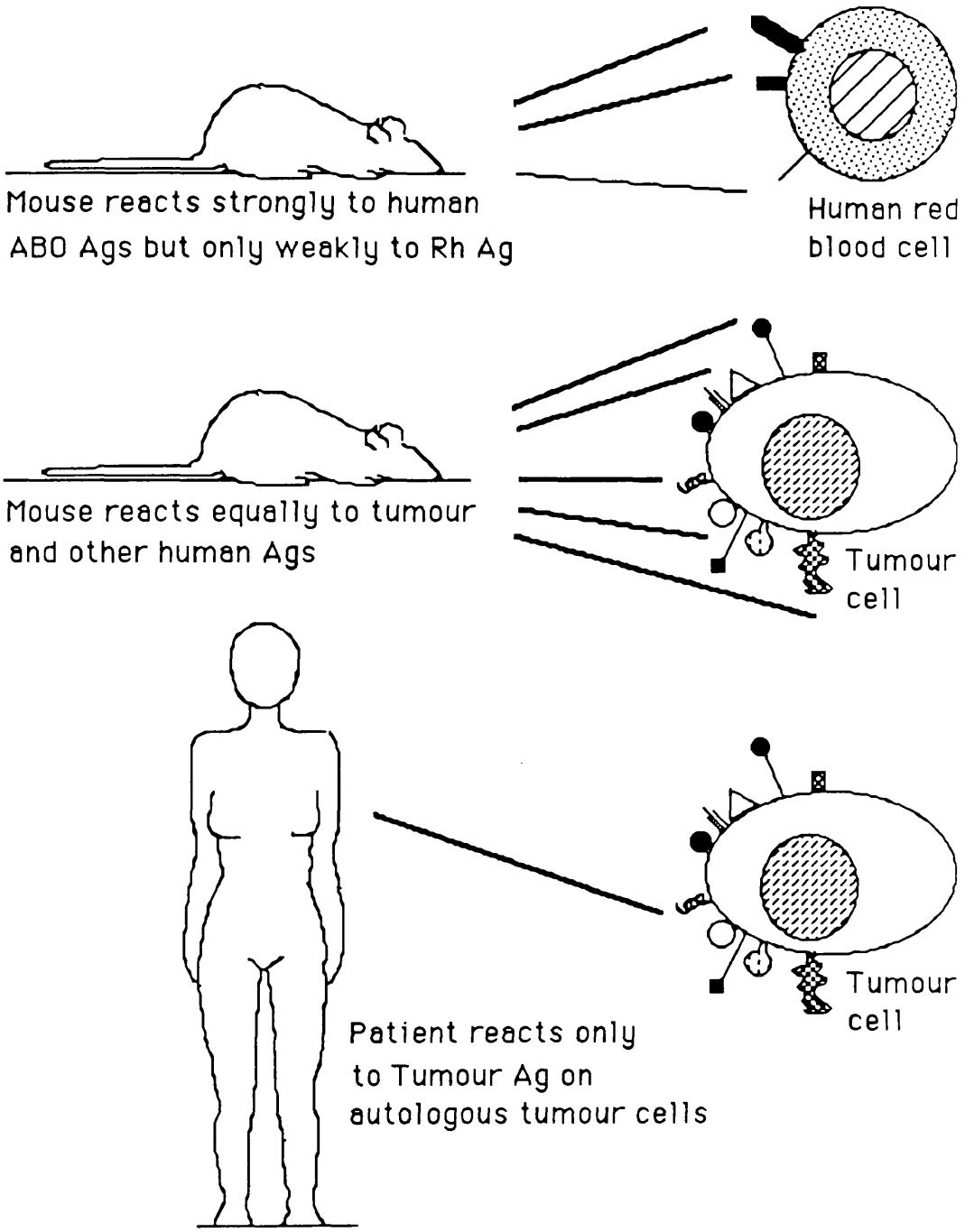


Fig. 65 The humoral immune response of patients to their autologous tumour cells is likely to be more specific than that raised in mice who mount a strong response to all human antigens on malignant and normal cells.

[400], human monoclonal antibodies have, as yet, made little impact on the diagnosis or therapy of breast cancer.

There are several reasons that may account for the failure to develop human monoclonal antibodies and, as no specific tumour antigens have yet been identified, one possibility is that there is no humoral immune response to immortalize. However, the results presented in the previous chapters would suggest this is not the case.

The greatest obstacle to the development and assessment of human monoclonal antibodies is the technical difficulty of producing them and, because of this, their potential has not yet been fully explored.

The different methods and their problems have been extensively reviewed [317, 211, 66, 67] and amount to three major difficulties.

Firstly, the source from which the B lymphocytes should be harvested.

How these lymphocytes can be immortalized and their production of useful immunoglobulin maintained.

Thirdly, how they should be selected to produce human monoclonal antibodies which will be specific for breast carcinoma.

SOURCE OF B LYMPHOCYTES

Due to the ease of harvesting, some groups have used peripheral blood lymphocytes but the antibodies produced from this source of B cells have tended to be of poor specificity with widespread cross-reactivity to other antigens [65, 66, 97, 211]. Olsson [317] suggested that the lymphocytic infiltrate within tumours could also be used to produce monoclonal antibodies but as this appears to consist largely of T lymphocytes it is unlikely that sufficient B cells would be obtained.

Several groups have suggested that the regional lymph nodes are likely to contain the greatest number of relevant B lymphocytes [160, 79, 76, 317, 80] and these have been used by most groups [385, 3, 231, 409] while Smordinsky *et al* [388] used lymphocytes harvested from a malignant pleural effusion. Unfortunately, lymph nodes are not always easily available and, in some centres, chemotherapy for breast cancer is given preoperatively, making it difficult to obtain lymph node lymphocytes which have not been damaged by cytotoxic therapy. From the results presented in the previous chapters it can be seen that the axillary lymph nodes of many patients with breast cancer contain a large population of B lymphocytes committed to the production of IgG. As this is suggestive of a mature humoral response, we used the

nodes as our source of B lymphocytes and selected out the IgG bearing cells to be immortalized for monoclonal antibody production.

IMMORTALIZATION

The second problem of human monoclonal antibody production, and indeed the greatest, is the immortalization of antibody producing lymphocytes. While the production of murine monoclonal antibodies has changed little since the initial success of Kohler & Milstein [235], the production of human antibodies has been tackled in several different ways but as yet no satisfactory method exists. Although new methods are currently being developed, the three most commonly available were considered here to assess the feasibility of immortalizing the humoral response and thus allow study of its specificity.

EBV transformation

This method was used by Steinitz *et al* [394] to produce the first human monoclonal antibody.
The human B lymphocytes are transformed by Epstein-Barr virus (EBV) which is obtained from the culture supernatant of the marmoset cell line B95-8 [278]. EBV is

a herpes-type DNA virus which preferentially infects human B lymphocytes and can insert its DNA into the human genome causing the cell to become activated and, in some cases, immortalized. Although EBV can infect all B lymphocytes, only a small number are transformed and, as yet, controversy remains as to which cells become immortalized. While it has been suggested by one group that EBV immortalizes small resting B lymphocytes which are IgG positive [6], another group suggests that it is large, activated, IgM positive cells which are immortalized and that this is dependent on entry into the cell cycle [70]. It has also been suggested by one group that those cells which transform are those which secrete immunoglobulin [390] while others found the opposite [428, 85, 13]. This latter group found that, of B lymphocytes infected with EBV and carrying the EBV nuclear antigen (EBNA), only those bearing the CD23 B cell activation marker became immortalized. When the B lymphocytes were separated into CD23 positive and CD23 negative cells the vast majority of cells positive for cytoplasmic immunoglobulin were in the CD23 negative group. A further problem of EBV transformation is the very poor cloning efficiency of lymphoblastoid lines [211] and although most of the cells do not transform, many cells are stimulated to divide and make selection of the transformed cells difficult. This explains the

difficulty in developing longstanding antibody producing cell lines from EBV transformed B lymphocytes. Even when this can be achieved the output of immunoglobulin from these cells is very low.

Our main aim, however, was not to produce monoclonal antibody lines but to set up short term cultures of IgG secreting B lymphocytes, harvested from the axillary lymph nodes, and assay the supernatants for autologous tumour binding. We therefore chose this method as our main approach because of its simplicity.

Fusion

Fusion with an immortal cell line, the method for producing murine monoclonal antibodies, involves taking stimulated splenic B lymphocytes, from a mouse immunized with the antigen of interest, and fusing them with cells from a non-secreting mouse myeloma cell line. The equivalent method in human monoclonal antibody production would be to harvest the B lymphocytes from a patient, which have been naturally stimulated by her tumour, and fuse them with a non-secreting human myeloma cell line. This was first attempted by Olsson & Kaplan [315], who fused human B cells with a human myeloma line but, unfortunately, the myeloma they used secreted IgE and was difficult to handle.

Although a non-secreting human myeloma cell line does

exist [202] most lines secrete their own immunoglobulin and therefore produce a scrambled mixture of the myeloma immunoglobulin and the desired antibody.

While they are capable of a very high output of antibody, the human myeloma cell lines that have been developed grow so poorly in culture and are so difficult to clone that this largely precludes their use as fusion partners [423, 79, 80, 316]. The other type of human fusion partner available is the lymphoblastoid cell line. These cell lines are usually produced from lymphocytes immortalized after infection with EBV. All of these cell lines secrete their own immunoglobulin despite the fact that they are generally poorly developed for immunoglobulin production, with sparse rough endoplasmic reticulum or golgi apparatus. They are, however, easier to grow than myelomas, although they clone poorly, and the majority of human fusion partners for anti-tumour human-human hybridomas have been lymphoblastoid cell lines [382, 79, 160, 52, 80, 3, 231, 409]. Due to the lack of adequate human fusion partners non-secreting mouse myelomas have also been used in producing human antibodies [469, 1, 181, 192, 385, 388]. The resultant human-mouse hybrids secrete only the human antibody, and in greater quantities than the lymphoblastoid fusions, but unfortunately they are unstable because of the two sets of genes within the

cell. After some time the human genes are preferentially extruded [241, 414] including chromosome 2, which codes for the light chain of the immunoglobulin, while chromosome 14, which codes for the heavy chain, is retained [90]. This loss of the human genes leads to a loss of human immunoglobulin secretion and antibody production. To salvage secretion the hybridoma must be recloned every few months and even this strategy does not prevent the loss of many useful monoclonal antibodies, although stability, with recloning, up to 36 months has been reported [192].

In an attempt to overcome these difficulties heteromyelomas have been developed but the antibody secreting cell lines produced from them have also tended to be unstable [68]. As yet therefore, the search continues for an ideal fusion partner for human monoclonal antibody production.

Due to its better handling and cloning a non-secreting mouse myeloma, P3-x63 Ag8.653, was chosen as the fusion partner for this study.

EBV transformation and backfusion

Kozbor *et al* [239] suggested combining both of the above techniques in an attempt to gain the advantages of both methods while avoiding some of the disadvantages. The biggest advantage of EBV transformation followed by

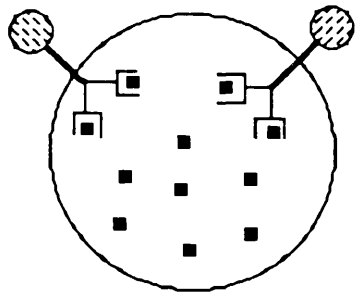
backfusion is that the fusion rate increases by a factor of five [240, 196]. This is due to the polyclonal activation of the B lymphocytes which causes them to pass through the DNA cell cycle faster and therefore increases the chances of successful cell fusion. When the three methods were compared using either lymph node lymphocytes or peripheral blood lymphocytes the greatest success was achieved using combined EBV infection and backfusion with lymph node lymphocytes [470]. This method does not however prevent the loss of the human genes from the mouse myeloma cell and with it the loss of antibody secretion. These antibody producing cell lines are therefore no more stable than those produced by fusion alone.

ASSAY AND SELECTION

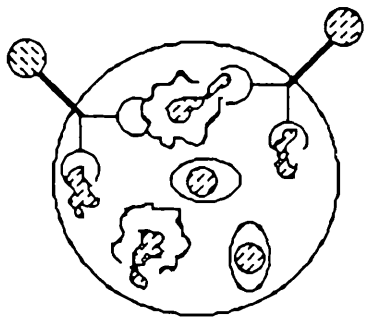
The commonest assay used to identify antibody binding is the enzyme linked immunosorbent assay (ELISA) which involves binding the antigen under study to a fixed surface, usually a multiwell plastic tray, adding the supernatant being tested and staining for a positive reaction using an enzyme linked to an anti-immunoglobulin antibody. This assay is quick and efficient when the antigen of interest is small and

highly purified but when whole tumour cells are used they very quickly become damaged and this allows access of the antibody, or supernatant, under test to the intracellular and cytoskeletal proteins which cross-react with many antibodies [155, 66, 97, 156] leading to false positive results (Fig. 66). This problem has led to the identification of many monoclonal antibodies which appeared to react with tumour cells but were later found to cross-react with a wide range of antigens and normal tissues [156, 385, 231, 409]. Antibodies were produced, from normal volunteers, which reacted with toxins such as ricin, which is lethal in nanogram amounts, to which the subject could not have had any previous exposure [97]. This problem of cross-reactivity or monoclonal multispecificity has only been appreciated in recent years and appears to be worst with IgM monoclonal antibodies which constitute the initial response to an antigen and are polyvalent [66] while bi-valent IgG antibodies, which develop after isotype switching, represent a more mature secondary immune response and have higher affinity, with less multispecificity (Fig. 67). Cross-reactions still occur even with antibodies of the IgG class and care must still be taken in their assay.

Immunohistochemical assays, which allow direct vision of supernatant binding on frozen sections of tumour, are



Specific, high affinity binding to purified antigen



Antibodies bind inappropriately to the DNA and cytoskeletal proteins of damaged tumour cells

Fig. 66 While ELISA assays are fast and efficient in the testing of antibodies to highly purified antigens, the lack of a defined tumour antigen and the access of antibodies to the DNA and cytoskeletal proteins of damaged tumour cells, leads to the false identification of antibodies as tumour specific.

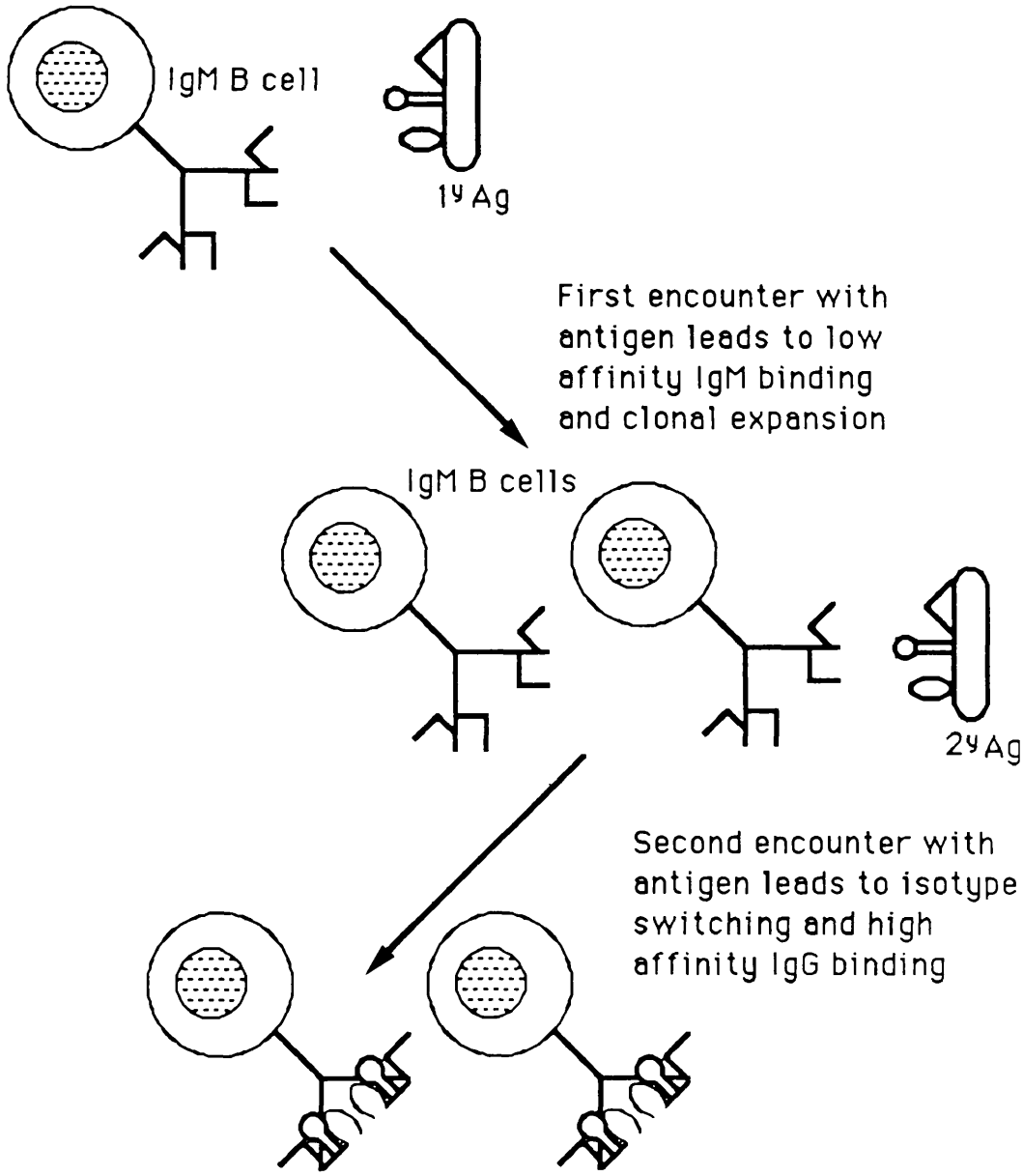


Fig. 67 While IgM is produced during the initial humoral response to antigen, IgG is produced after isotype switching and maturation of that response and binds the antigen with greater affinity and specificity.

more informative with regard to the degree and distribution of antibody binding within the tumour. Antibodies selected by this method can be further assayed using the live immunofluorescence assay in which live tumour cells are used to assay the antibody, or supernatant, with a fluorescein conjugated anti-human IgG second antibody showing any positive binding when viewed by fluorescent microscopy. Non-specific binding to the intracellular proteins in dead cells is avoided by using a Hoechst preparation which stains the nuclei of dead cells blue, allowing their identification and exclusion from the assay.

PRESENT STUDY

In an attempt to isolate and study the humoral immune response, suggested by the results presented in the previous chapters, the IgG producing B lymphocytes from the axillary lymph nodes of breast cancer patients were harvested, immortalized using one of the three methods mentioned above and assayed on frozen sections of autologous tumour.
It was also hoped that by using these preselection and assay methods, to isolate the humoral immune response of breast cancer patients, it might also be possible to

clone an IgG monoclonal antibody which would be specific for breast tumour and have little cross-reaction with normal tissues.

PATIENTS AND METHODS

SELECTION OF B LYMPHOCYTES

Lymph nodes were obtained aseptically from 35 patients undergoing definitive surgery for breast cancer which included excision sampling of the axillary lymph nodes. Half of each node was sent for routine histological examination while lymphocytes were harvested from the other half as described in chapter 2. Briefly, upon receipt of the lymph node sample, it was washed in RPMI 1640 medium and, after transfer to a fresh petri dish, the cells were teased out using a needle and scalpel. This method left the connective tissue structure of the node behind, thus reducing the contamination by fibroblasts. The cells spilled from the node were pipetted up, washed and resuspended in 10ml of RPMI medium. This cell suspension was layered over an equal volume of Ficoll Hypaque and the lymphocytes harvested by density gradient separation. These lymphocytes were again washed, resuspended in 10ml of RPMI medium and counted in a haemocytometer. A process of negative selection was then used to isolate the IgG producing B cells, removing first the T lymphocytes and then the IgM producing B cells (Fig. 68).

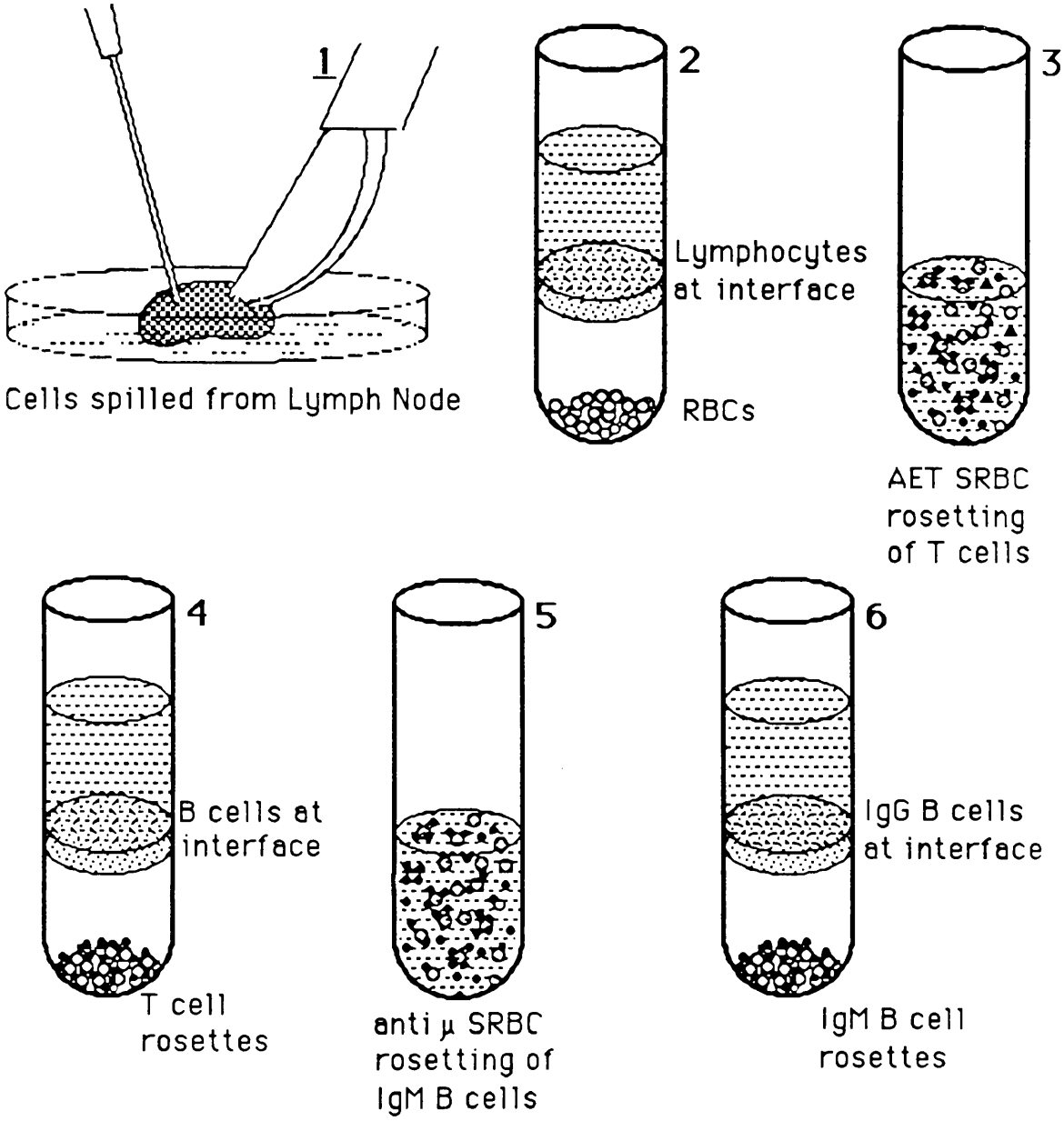


Fig. 68 After mechanical disaggregation of the lymph node (1), the lymphocytes were harvested by density gradient separation (2). The IgG bearing B cells were isolated, using negative selection, by rosetting out the T cells and IgM B cells (3-6). These IgG B cells were then immortalized by EBV transformation or cell fusion techniques.

The lymphocytes harvested from the lymph node were centrifuged and resuspended in 2% aminoethylisothiouronium bromide (AET) coated sheep red blood cells (SRBCs) to rosette out the T lymphocytes. 1ml of AET SRBCs was added for each 10^7 lymphocytes being treated, along with the same quantity of RPMI medium and foetal calf serum (FCS). The test tube was centrifuged at 100 G for five minutes and left on ice for 90 minutes.

After this time the tube was rocked gently to resuspend the rosettes, which could be clearly seen under the microscope, and the rosetted cells were removed by density gradient separation over Ficoll Hypaque. The unrosetted lymphocytes, which consisted largely of B cells, were removed from the interface, washed and recounted.

The IgM bearing B lymphocytes were also removed by rosetting but this time the SRBCs were coated with an antibody against the u chain of IgM immunoglobulin. The remaining lymphocytes were retrieved in the same way as before, using density gradient separation. These remaining lymphocytes had a greatly increased proportion of IgG producing B lymphocytes which was confirmed on several occasions by staining some of the suspension with a FITC conjugated anti-human IgG antibody and viewing under a fluorescent microscope. These IgG

producing B lymphocytes were then immortalized.

Preparation of AET sheep red blood cells

102mg of 2-amino-ethylisothiouronium bromide (AET) was weighed out and dissolved in 10ml of distilled water. This was adjusted to pH 9 using 5 molar (M) sodium hydroxide and then filter sterilized. The sheep red blood cells (SRBCs) were spun down out of fresh sheep blood and 1ml of the packed cell volume removed and washed twice in RPMI medium. 4ml of the AET solution was added to the washed SRBCs and incubated for 20 minutes at 37°C. The SRBCs were then washed five times in RPMI medium and diluted with 9ml of RPMI medium to give a 10% suspension of AET coated SRBCs. This was stored at 4°C and used within one week. The suspension was diluted to 2% prior to its use in rosetting out the T cells from lymph node lymphocytes.

Preparation of anti-u chain coated sheep red blood cells

2ml of SRBCs were washed three times in 0.15M sodium chloride and 0.5ml of the packed cells removed and diluted with 4ml of 0.15M sodium chloride.
To this was added 0.5ml of a 1:200 dilution of rabbit anti-human IgM (u chain) antibody (Dako), bringing the total volume to 5ml.
An aliquot of hydrated chromic chloride was brought to

pH 5 using molar sodium hydroxide and diluted to 1:100 by taking 0.1ml of chromium chloride and adding 9.9 ml of 0.15M sodium chloride. The solution was then filter sterilized and 5ml of this was added very slowly to the suspension of antibody and SRBCs. This suspension was then placed on a mixing plate for 20 minutes.
After this period the SRBCs were washed three times in phosphate buffered saline (PBS) and resuspended in 4.5ml of PBS to give a 10% suspension which was later diluted to 2% before being used to rosette out the IgM bearing B lymphocytes from the lymph node cell suspension.

IMMORTALIZATION

EPSTEIN-BARR VIRUS TRANSFORMATION

The suspension of IgG producing B lymphocytes, obtained by the selection method described above, was spun down and the pellet resuspended in 1ml of EBV suspension. The virus was obtained by ultracentrifugation of culture supernatant from the B95-8 marmoset cell line. The suspension was incubated overnight at 37°C and the following morning, RPMI medium was added to the virus suspension and the cells centrifuged to wash out any viral particles. After repeating this last step, the

IgG producing lymphocytes were resuspended in lymphocyte culture medium and plated out on 96 well plates. IgG secreting B lymphocytes from 22 patients were treated in this way.

CELL FUSION

Using this approach the IgG bearing B lymphocytes were fused with a non-secreting mouse myeloma line P3-X63 Ag8.653. The B lymphocytes had either been EBV transformed or were cultured for 5 days in a flask of lymphocyte medium which contained 3ng/ml phorbol myristate acetate (PMA), with added T cell conditioned supernatant for the last three days. The B lymphocytes were harvested and approximately $5X10^7$ human and mouse cells were used.

A small control sample of each cell type was taken and plated out to assess how quickly the selective culture medium killed the unfused cells. The two cell types were mixed, centrifuged together at 600 G for 5 minutes and the supernatant drained off to leave the cell pellet. This was loosened and then slowly resuspended in 1ml of warm polyethylene glycol (PEG) while stirring gently for 3 minutes.

10ml of RPMI medium was added very slowly over ten

minutes with only 1-2ml being added in the first 5 minutes.

This cell suspension was incubated at 37° for 20 minutes, the cells were then spun down and the supernatant decanted off thoroughly to remove all the PEG.

After washing, the cells were resuspended in medium containing 0.1M hypoxanthine, 0.016M thymidine and 0.4uM aminopterin so that only fused cells might survive. If EBV transformed cells were used in the fusion, 10^{-5}M ouabain was also added to destroy unfused cells. The unfused parent cells should die out at 8-10 days as the fused clones begin to appear.

T cell conditioned supernatant

This is obtained by culturing the T cells which were removed from the lymph node lymphocyte suspension by AET SRBC rosetting. The T cells were released from the rosettes by lysing the SRBCs with distilled water and then harvested by density gradient separation over Ficoll Hypaque. The T cells were washed and resuspended in lymphocyte culture medium, at a density of 10^6/ml, containing PMA at a concentration of 10ng/ml and phytohaemagglutinin (PHA) at a concentration of 10ug/ml. After 48 hours of culture the cells were centrifuged and the supernatant collected for use in B cell cultures.

Lymphocyte medium

This consisted of 80% RPMI medium and 20% FCS with both insulin and transferriin added at a concentration of 5ug/ml. It was prepared in small volumes of 50ml to ensure that fresh medium was used at all times.

IMMUNOHISTOCHEMICAL ASSAY OF ANTIBODY SUPERNATANTS

This was performed on frozen sections of autologous tumour using one slide for each well to be assayed. The sections were cut on a microtome at 6u and dried for one hour in a box of silica crystals. The sections were fixed in acetone for fifteen minutes and placed in a humid box where all subsequent preparation was carried out. The sections were blocked for thirty minutes with bovine serum albumin (BSA) to reduce non-specific binding of the test supernatants and in particular to block the IgG present within the tumour.

50ul of test supernatant was placed on each slide, covering all of the tumour section, and incubated in the humid chamber for one hour (Fig. 69).

After this time the slides were rinsed with tris buffered saline (TBS) and 50ul of a 1:200 dilution of biotinylated anti-human IgG conjugate (Vector Laboratories, Peterborough) was added.

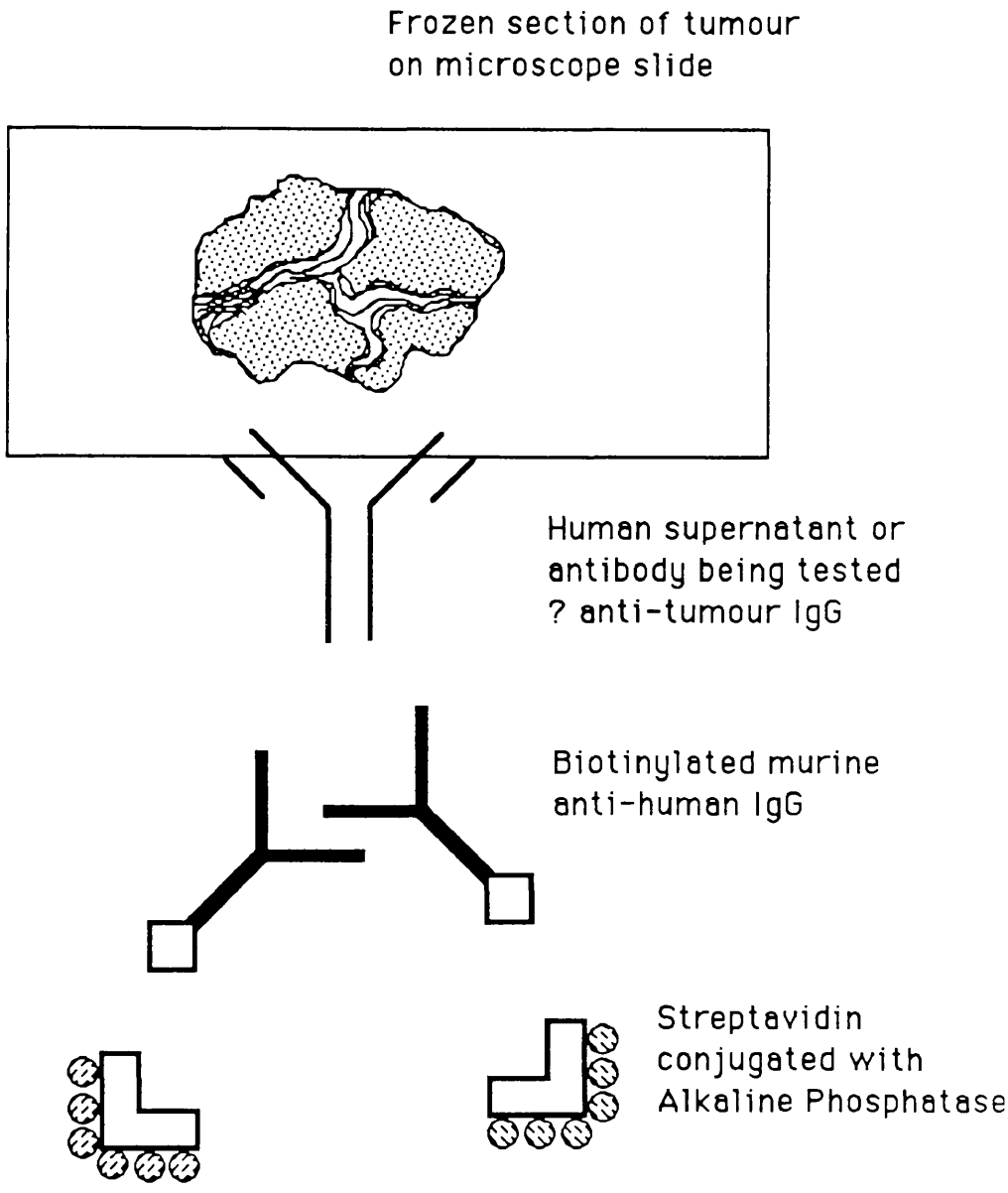


Fig. 69 The supernatants from the immortalized IgG B cells were assayed on frozen sections of autologous tumour using a biotin-avidin link between the murine anti-human IgG antibody and the alkaline phosphatase. After developing, IgG binding could be seen as small red granules when viewed under a light microscope.

This was incubated for one hour when, after rinsing the slides with TBS, 50ul of streptavidin bound alkaline phosphatase was added to each slide. The slides were again incubated for one hour in the humid chamber.
The slides were rinsed thoroughly with TBS and developed. The developer consisted of 4mg of napthol AS-MX phosphate, 10mg of fast red violet and 2.5mg of levamisole, the last of which inhibits intrinsic alkaline phosphatase in the tissue section. These were dissolved in 20ml of veronal acetate buffer and each slide was covered in an excess of developer and incubated until the remaining developer was seen to become opaque.
The slides were rinsed in tap water and left overnight in formal saline. The following morning, the slides were again rinsed in tap water and counterstained with haematoxylin and Scots tap water.
The sections were viewed under a standard light microscope and antibody binding was seen as crimson red granules.

STOCK SOLUTIONS

Scots Tap Water

7g of sodium bicarbonate and 40g of magnesium sulphate were dissolved in 1000ml of distilled water. A few thymol crystals were added as a preservative.

Tris Buffered Saline (TBS) pH 7.4

6.055g of tris hydrochloride (50mM) and 11.68g of sodium chloride were added to 1000ml of distilled water and brought to pH 7.4 with 0.1M hydrochloric acid.

Veronal Acetate Buffer

9.78g of sodium barbitone and 6.7g of sodium acetate were dissolved in 1000ml of distilled water and brought to pH 9.2 using 0.1M hydrochloric acid.

Chromic Chloride

5g of hydrated chromic chloride and 4.383g of sodium chloride were added to 500ml of distilled water to produce a 1% w/v solution. This was aged at room temperature for two weeks and then aliquoted to avoid repeated pH adjustment.

RESULTS

Lymph nodes from a total of 35 breast cancer patients were processed to remove the T cells and IgM producing B cells leaving the lymphocyte suspension enriched for IgG producing B cells. These lymphocytes were then immortalized by the three common methods discussed above and their supernatants assayed on frozen sections of autologous tumour using an anti-human IgG antibody to detect the binding of any IgG, present within the supernatant, to the tumour section.

EBV TRANSFORMATION

The IgG producing B lymphocytes from 22 patients were transformed using EBV. After the transformation, the growth period usually lasted between one to two weeks when expansion would slow and the cells would begin to deteriorate as the untransformed cells gradually died away. Lymphocyte cultures from six patients survived long enough to allow the supernatants to be assayed. Four out of the six lymphocyte cultures had wells from which the supernatant showed some IgG binding to the tumour sections (Figs. 70-73 in which "a" is the control and "b" includes the supernatant under test). None of these cell cultures survived the subsequent efforts to clone out the positive antibody secreting B

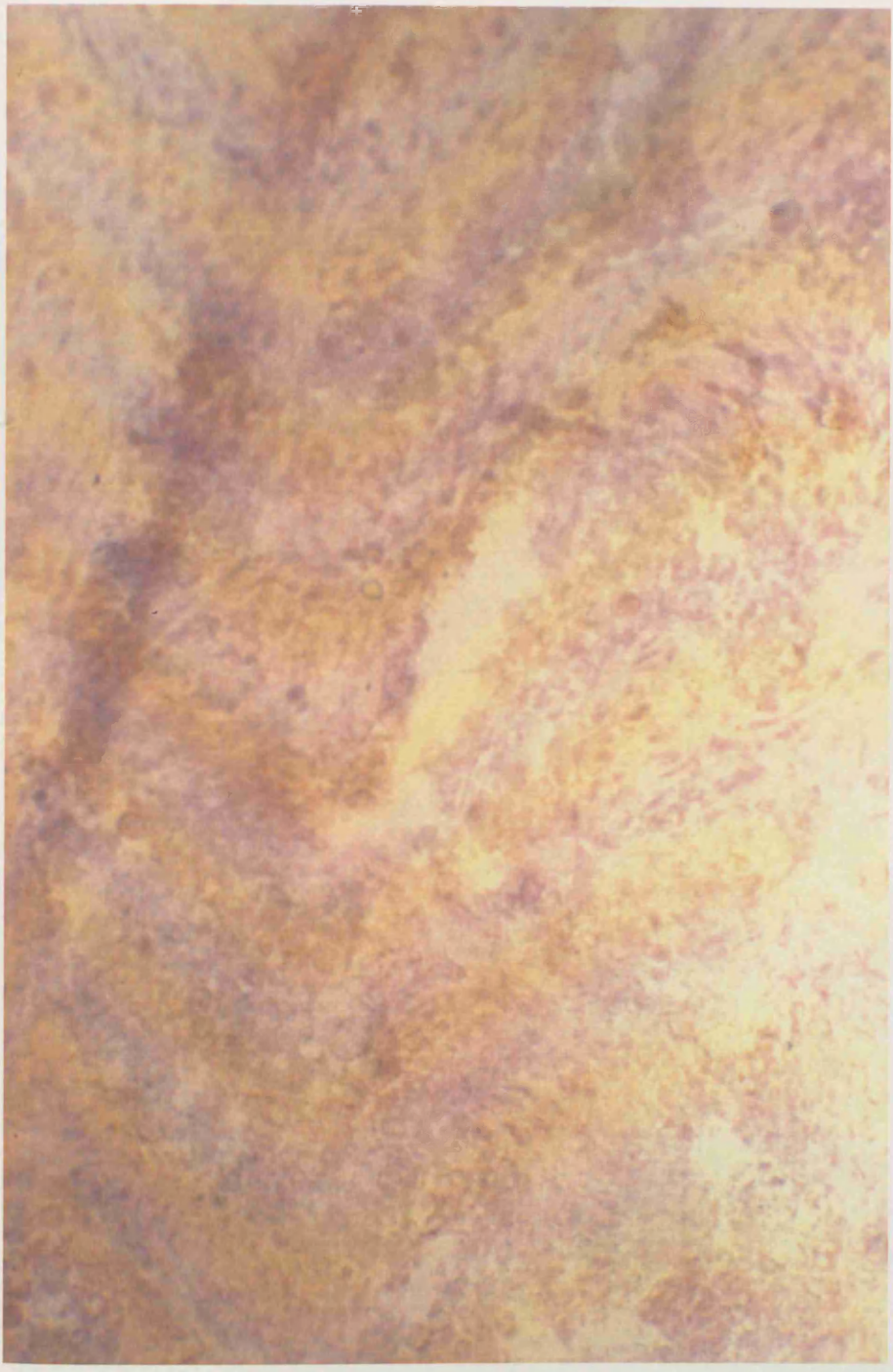


Fig.70a

Fig. 70b

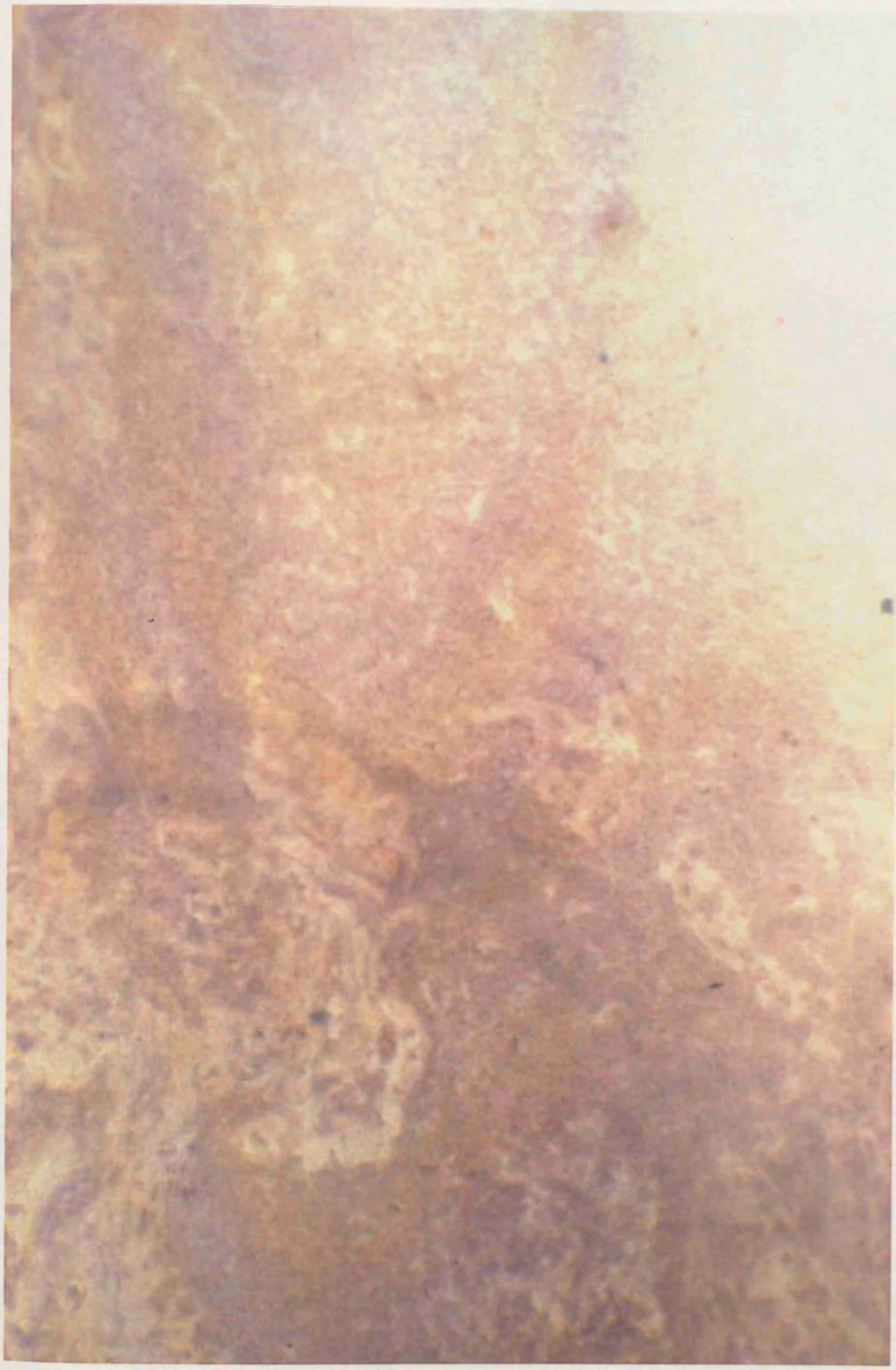


Fig. 71a

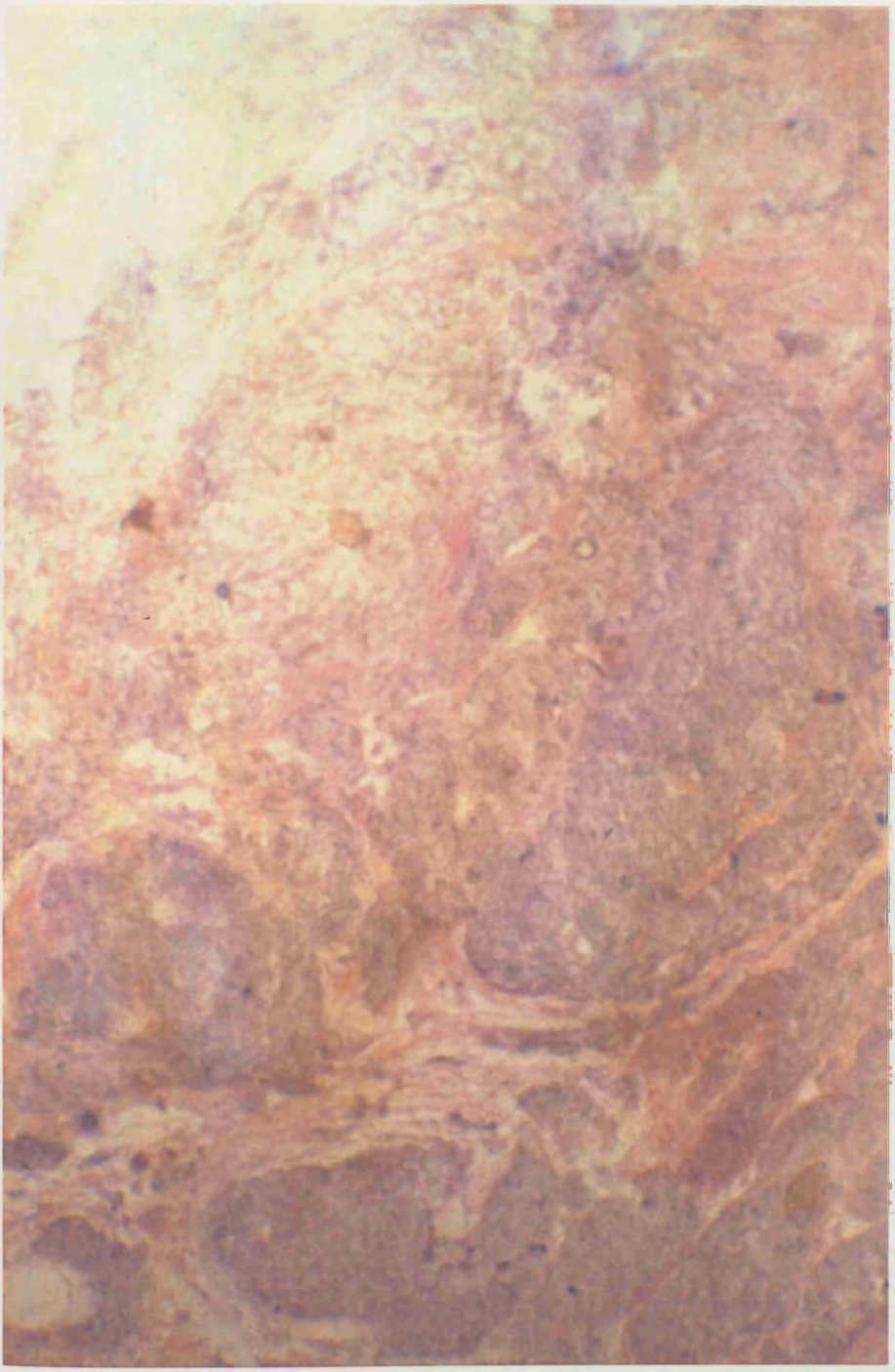


Fig. 71b

Fig. 72a

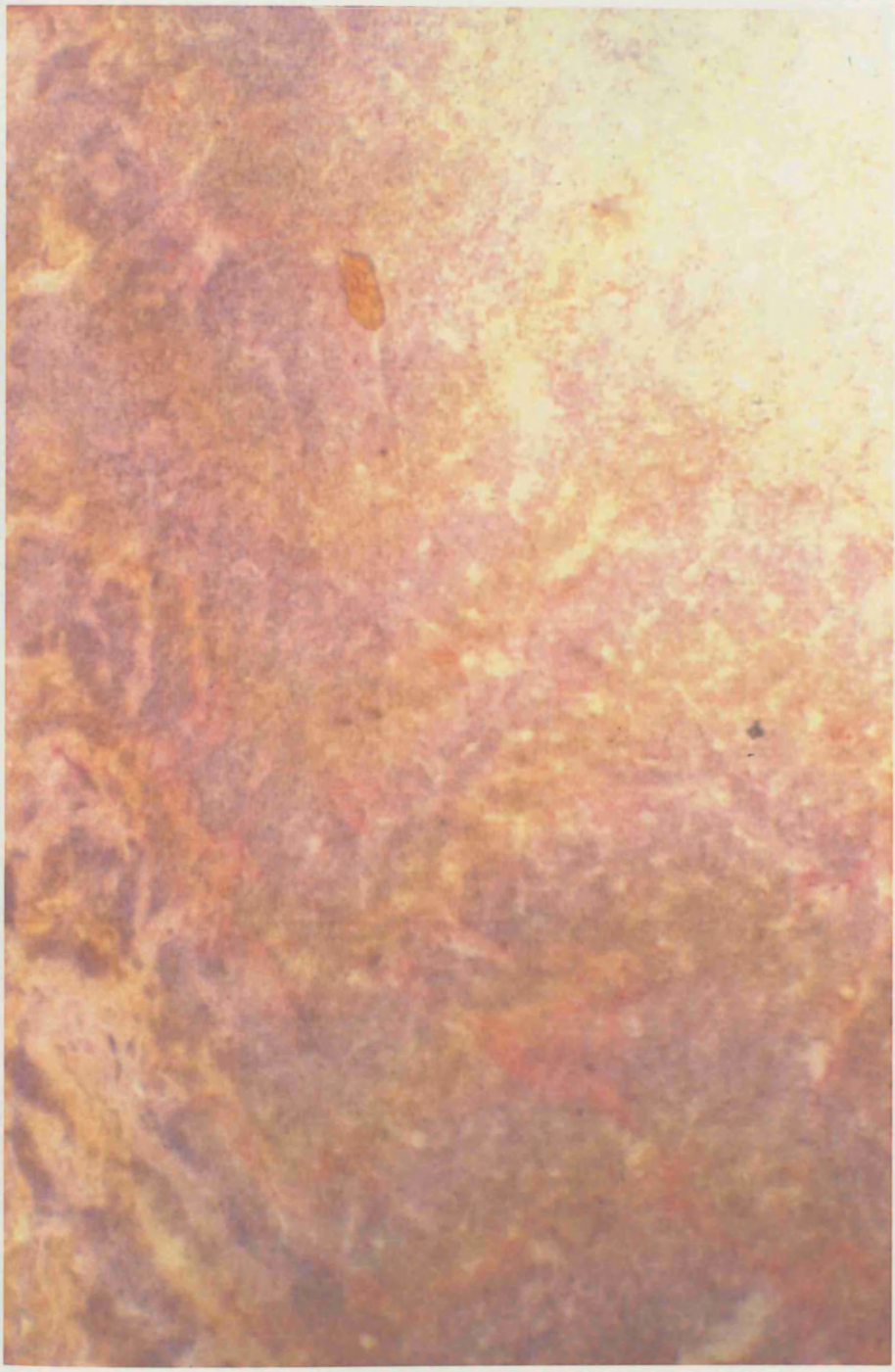


Fig. 72 b

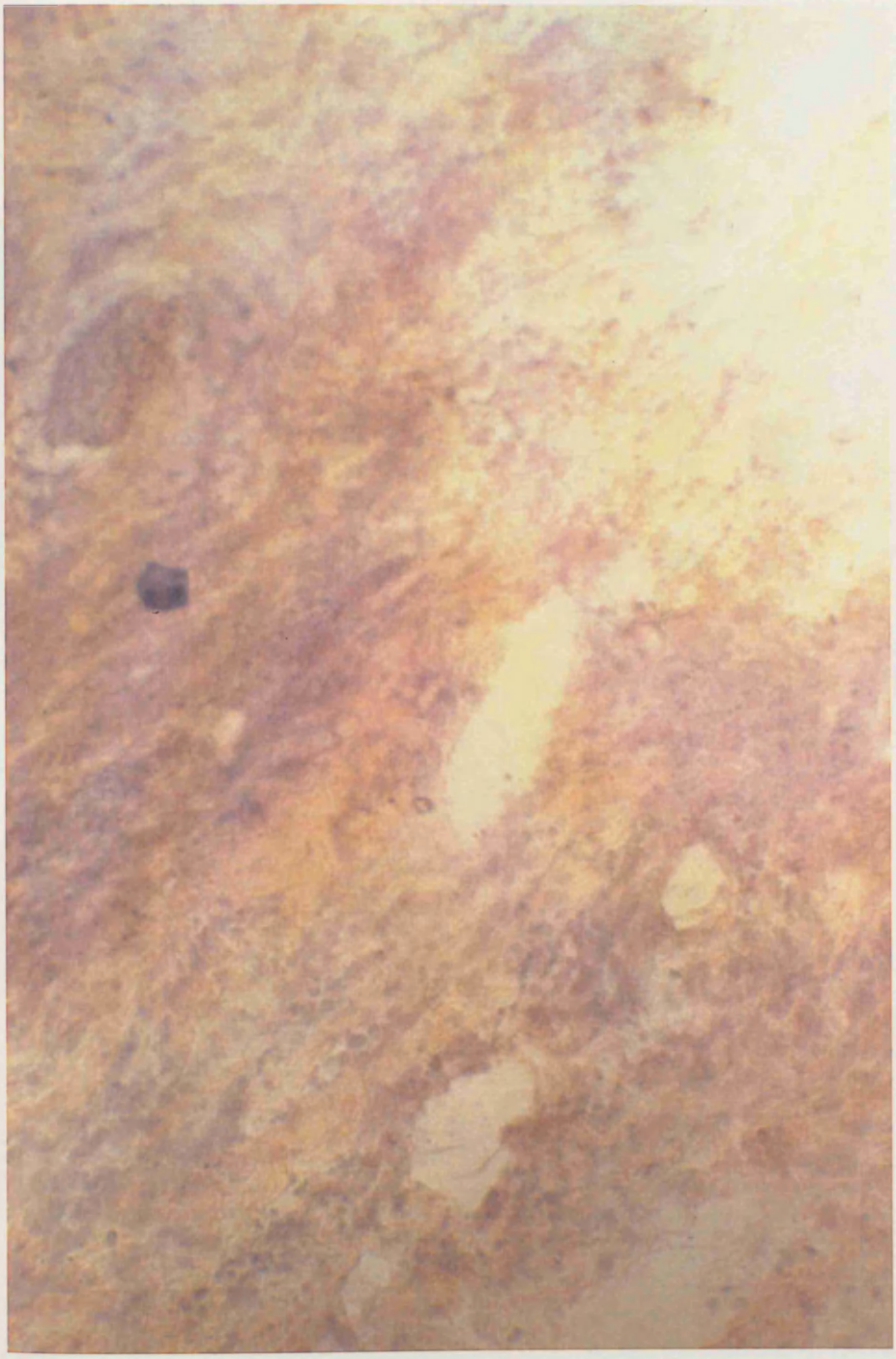


Fig. 73a

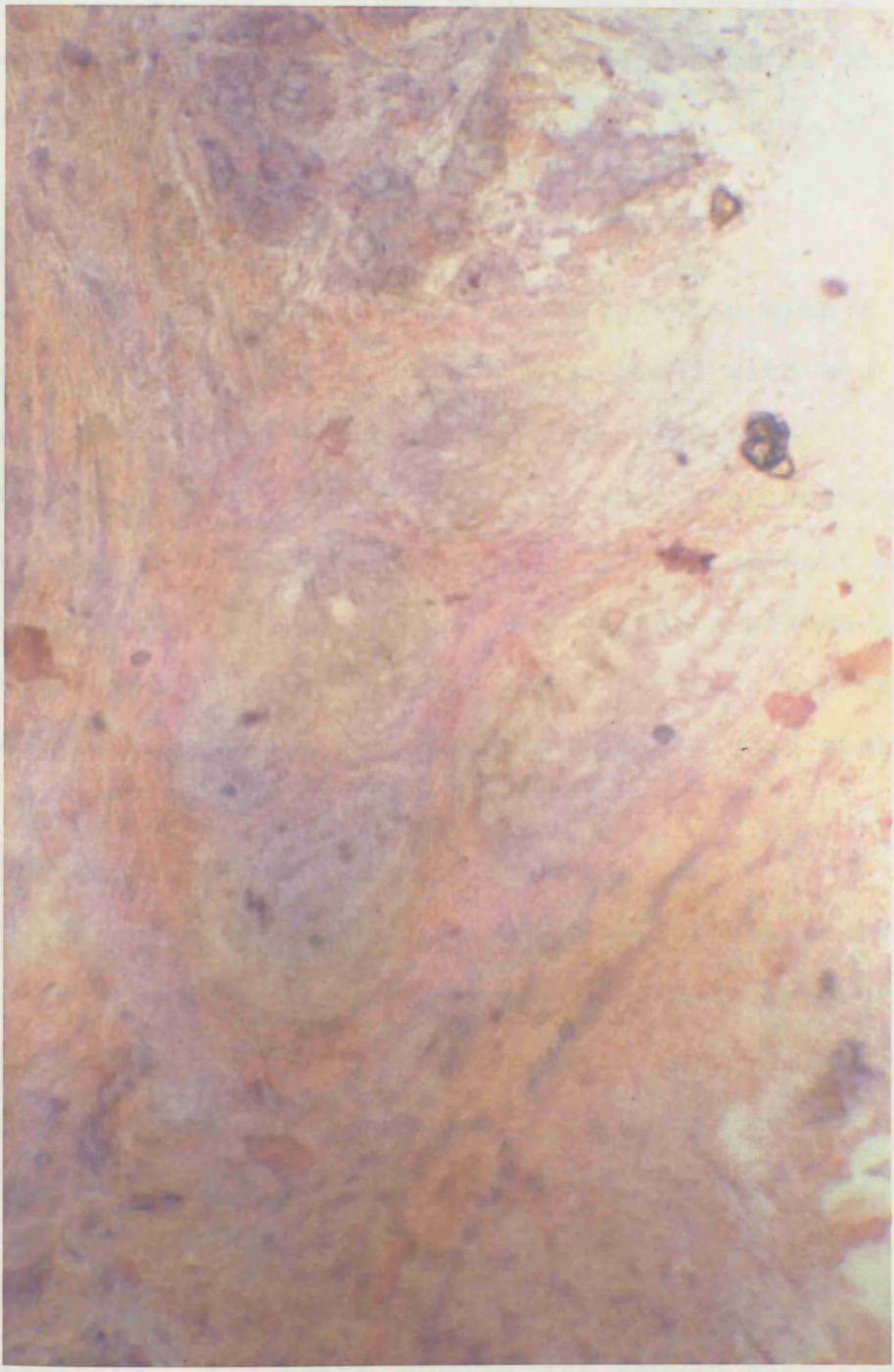


Fig. 73b

cells but died away when we attempted to establish monoclonal antibodies by seeding the cultures at a density of one cell per well. This problem was not overcome by the use of feeder layers such as mouse spleen cells.

FUSION

In four cases, the B cells were cultured with T cell conditioned supernatant and then fused with the non-secreting mouse myeloma P3-X63 Ag8.653. Unfortunately none of these cultures even survived long enough to allow assay of the cell supernatants on autologous tumour. Two of the cultures became contaminated with mould in the incubators and the other two cultures died away without fused clones appearing.

EBV TRANSFORMATION AND BACKFUSION

The B cells from nine patients were processed by EBV transformation followed, one week later, by backfusion. Only two of these survived to be assayed on autologous tumour sections. One culture had a few wells with staining which included the nests of tumour cells (Fig. 74; a = control, b = test) but antibody secretion was lost during the cloning stages, with later assays being negative. The other supernatant showed mild staining of the tumour stroma (Fig. 75; a = control, b = test).

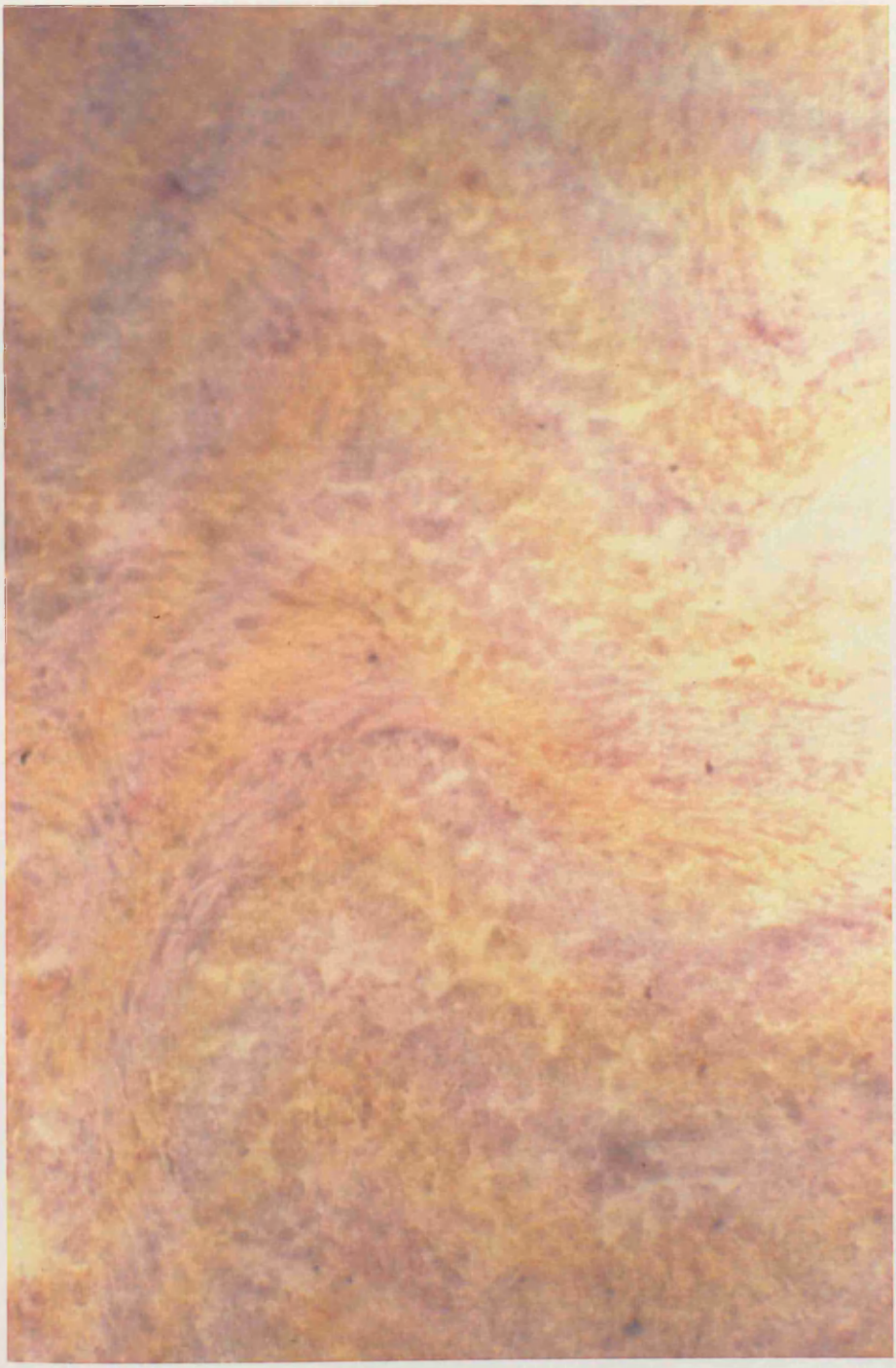


Fig. 74a

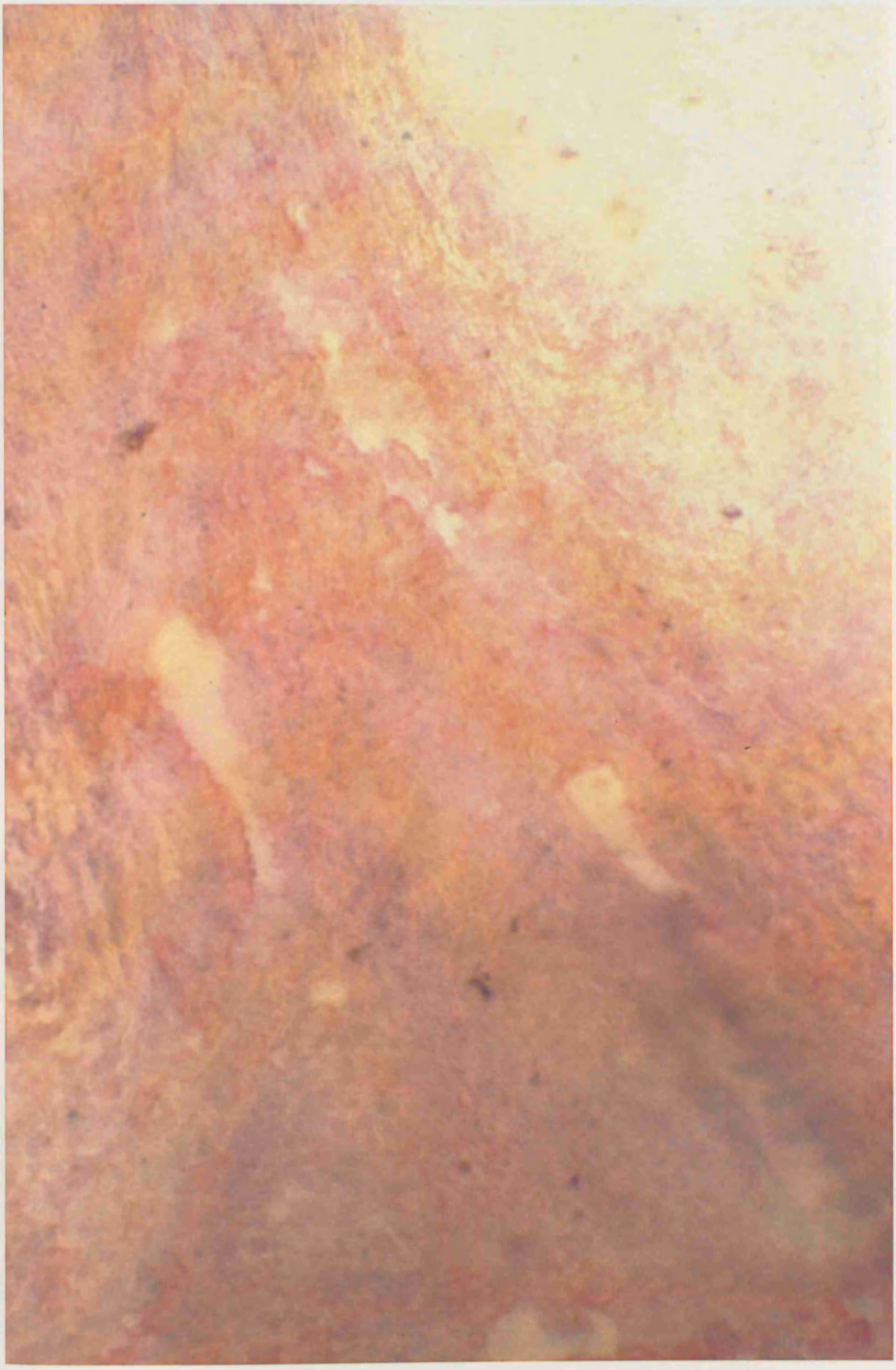


Fig. 74 b

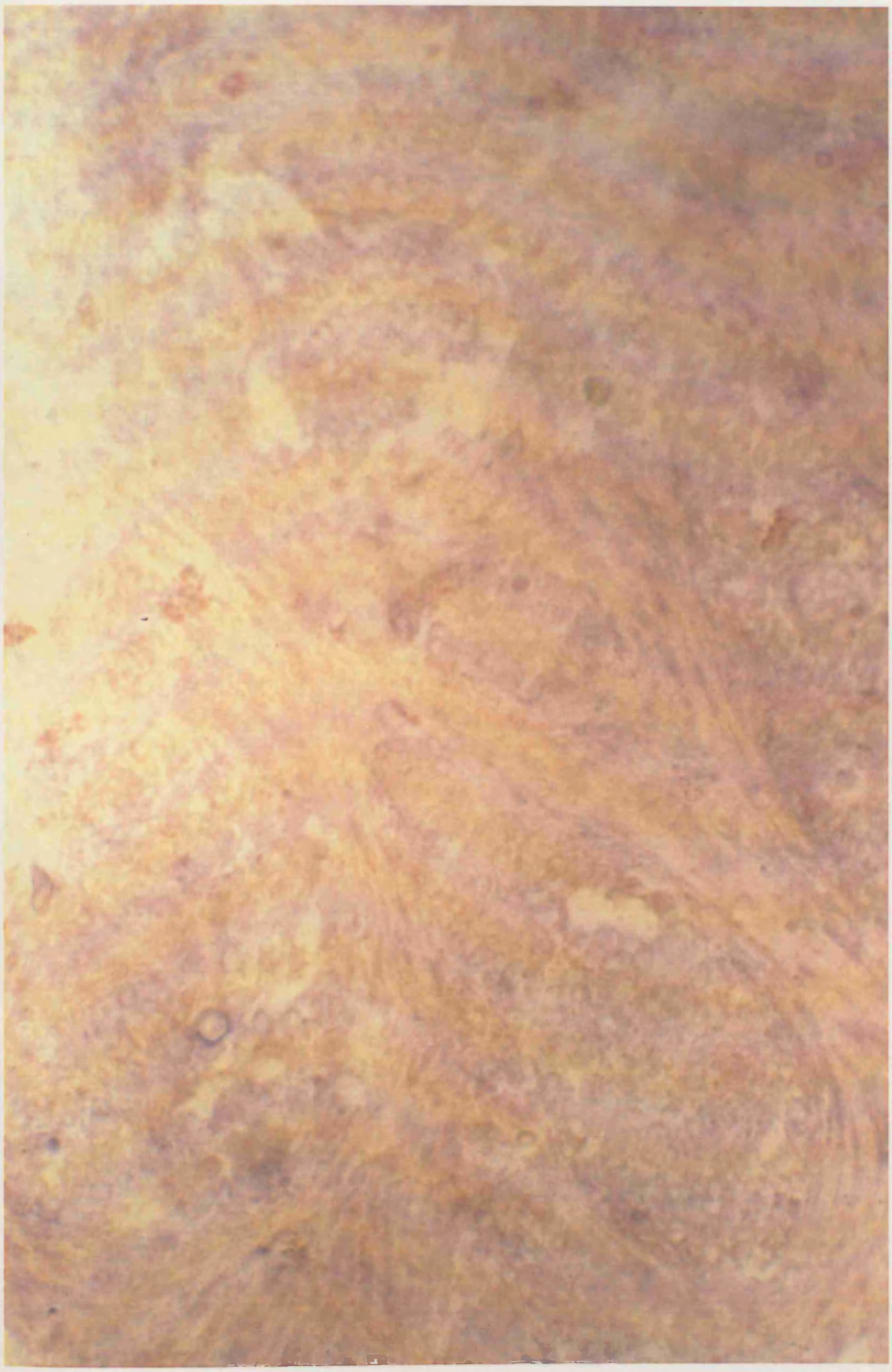


Fig. 75a

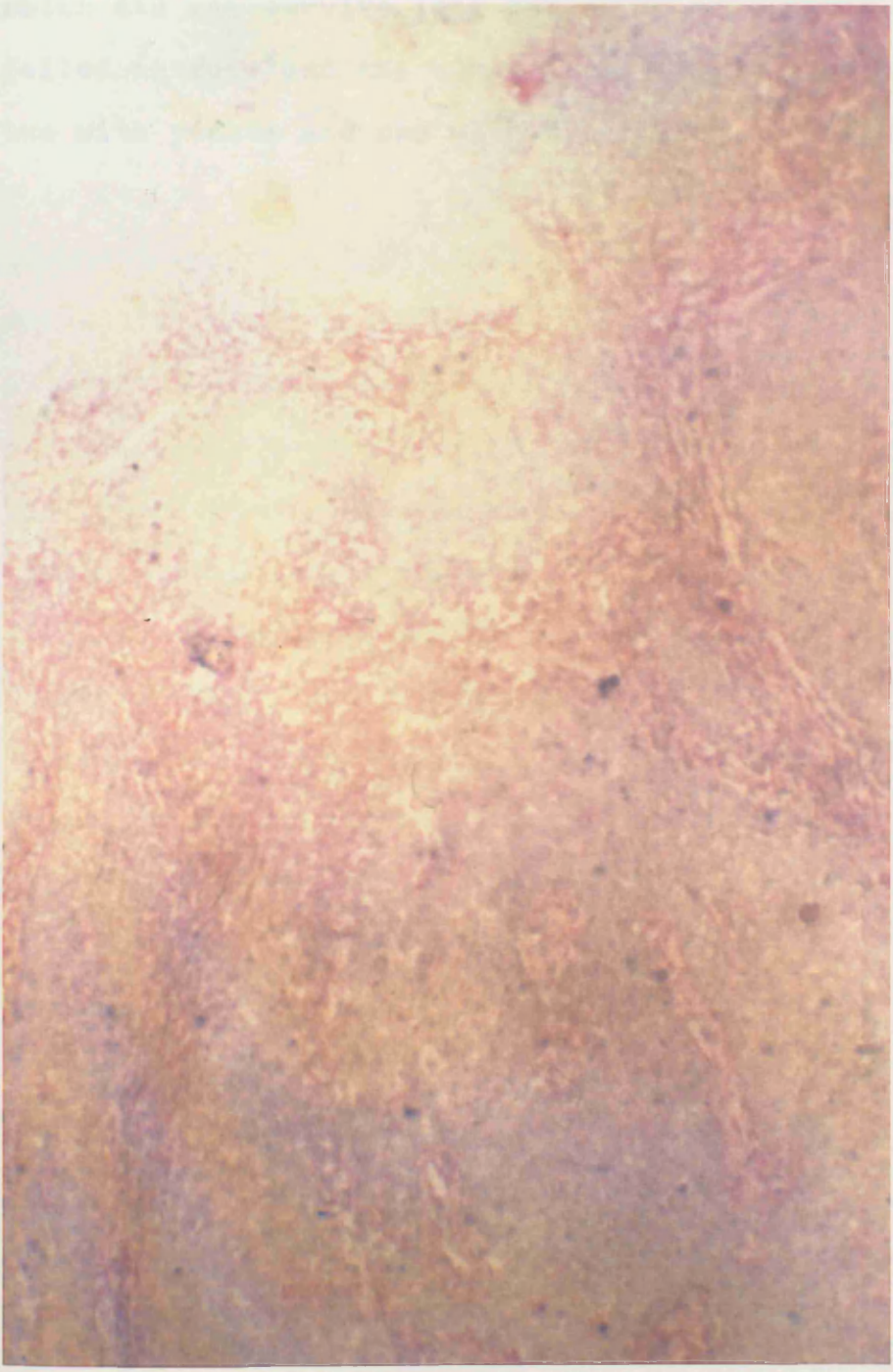


Fig. 75b

These cultures also, did not survive cloning due to cell death at low cell densities. Of the seven cultures which did not survive long enough to be assayed, four failed to fuse and the other three became contaminated, two with yeasts and one with fibroblast overgrowth.

DISCUSSION

EBV TRANSFORMATION

This method alone has not been applied to the production of human monoclonal antibodies from breast cancer patients. In our experience, although we could achieve good initial activation, and possibly transformation, it was difficult to clone any of these cell lines. Six of the transformed lines were assayed and four of these showed some positive staining on autologous tumour, which is in keeping with the results presented in the previous chapters and suggests there is some anti-tumour humoral response present in these patients. As we were unable to establish stable antibody clones, it was not possible to assess this response in more detail. It was particularly notable that the majority of supernatants appeared to preferentially stain the tumour stroma rather than the cell nests which is similar to the distribution found in studies of autologous IgG staining, using immunohistochemical techniques [106, 208]. EBV transformation has been applied more often to the isolation of serum antibodies such as those against Rhesus factor [84, 274, 146, 163, 243, 244, 420, 251] and tetanus toxoid [238], where murine antibodies are

inappropriate because the desired response is swamped by that to the major human antigens such as the ABO blood group antigens. In this field also, the success has been rather limited.

FUSION

This is the most common method which has been applied to the production of human monoclonal antibodies from patients with breast cancer. Like ourselves, most groups have used lymphocytes from the axillary nodes of breast cancer patients to fuse with an immortal cell line. While the early groups used non-secreting mouse myelomas as their fusion partners [373, 469, 415, 79, 205], some more recent projects have used human fusion partners [382, 79, 381, 399, 231, 388, 3, 385, 409]. These groups had some initial success in producing human monoclonal antibodies to breast carcinoma, three of which were of the IgG class, eight were of the IgM class, while two produced both and in one report the immunoglobulin class was not specified.
Many of these hybridomas, however, have not remained stable and while two are still being used in studies of tumour antigen [205, 399], none have, as yet, made any clinical contribution to patient management.

In this study we used only mouse myeloma fusion partners because of their greater tolerance and easier handling but none of the fusions were successful and so no assays were performed in this group.

EBV TRANSFORMATION AND BACK FUSION

This method has been applied by only one group working with breast cancer patients since its introduction by Kozbor *et al* [239]. They used peripheral blood as the source of lymphocytes rather than axillary lymph nodes [65].

The main advantage of this method is the higher fusion rate achieved because polyclonal activation of the human B lymphocytes causes them to pass through the cell cycle more quickly and thus increases the fusion opportunities [196]. Nine patients were processed by this method and two of these were sufficiently successful to allow assay of their supernatants. The cell line with the stronger staining survived the initial cloning but secretion was lost and recloning at low density was not successful.

FUTURE APPROACHES

From the results presented earlier in this work it would appear that some patients are mounting a fairly strong humoral immune response and this may be against their tumours. This response can only be fully characterized, however, when the technology of monoclonal antibody production has become sufficiently advanced to allow the simple and dependable immortalization of B cells from a large number of patients with breast cancer.

Conventional Methods

Easier immortalization might be brought about by the development of improved methods of cell fusion, such as electrofusion [473, 32, 105], and a human fusion partner with similar attributes to those used in murine antibody production.

A good fusion partner should be well adapted for high output antibody secretion, grow well in tissue culture and be easy to clone at low density to produce a monoclonal antibody.

It should produce no antibody of its own to avoid the production of a scrambled mixture of desired antibody with that of the fusion partner as this greatly reduces the affinity of the antibody produced. As yet such an ideal fusion partner has not been identified.

Gene Cloning Methods

Advances in human monoclonal antibody technology are, perhaps, more likely to come from the development of completely new approaches such as cloning heavy and light chain genes within an expression vector. The immunoglobulin chain genes of murine antibodies have been expressed in *Escherichia coli* [64, 53] and yeast [466]. However, only antibody fragments can be produced in *E. coli* and therefore, to obtain whole antibody, mammalian cells such as myeloma must be used [310, 216, 377, 365]. The plasmid, which carries DNA into the expression vector may be transfected by several methods but the most efficient of these is electroporation. By this method an electrical impulse causes the cell membrane to become temporarily permeable to the new DNA segment [10].

The most recent hope for advance comes from the development of the Polymerase Chain Reaction (PCR) [366]. With this method it is possible to amplify small segments of DNA, less than 5 Kilo base pairs (Kbp), providing sequences of 18 to 28 base pairs at either end are known [457].

There are still severe limitations of this new technology in its application to the production of human monoclonal antibodies .

The introductory segments must be sequenced by

conventional techniques before PCR is possible and while this is possible with an established hybridoma, because of the large number of identical cells available for sequencing, it limits its use with unimmortalized human B lymphocytes. One possible way round this would be to use the consensus sequences, from the Kabat and Wu database, which flank the variable regions as induction sequences [468]. This is, however, likely to select for the polyspecific IgM repetoire rather than the hypermutated IgG antibodies and may therefore produce antibodies of rather poor affinity.

A further limitation, which may gradually be overcome as the technique develops, is the small size of DNA segment which can be amplified. At present only 5 to 6Kbp can be amplified whereas larger genomic DNA is required to produce whole antibody.

Lastly there is a fairly high intrinsic error frequency when the heat stable Taq DNA polymerase is used and careful assaying will still be necessary. In the field of human tumours, this will still pose logistical problems.

Thus there is some way to go before PCR makes a major contribution to the development of human anti-tumour monoclonal antibodies. Its main use at present will be to amplify the variable region DNA of established but inherently unstable hybridomas.

CONCLUSIONS

It can be seen from our experience that immortalization of the humoral immune response, either for study or for the development of a human monoclonal antibody, remains difficult and requires great tissue culture expertise. Some of the difficulties we experienced were simply those inherent in long term tissue culture, such as cell death or the contamination of culture plates with micro-organisms, but even when these pitfalls were avoided it was found to be very difficult to immortalize the cells and then to maintain immunoglobulin secretion. The basic methodology for the production of human monoclonal antibodies remains flawed with no satisfactory technique, for the immortalization of immunoglobulin secreting lymphocytes in humans, which would be equivalent to murine hybridoma production.
If human monoclonal antibody production is to mirror the production of murine antibodies, we require a non-secreting human myeloma cell line which can be easily handled in tissue culture and cloning and will remain stable after fusion.
The other alternative is the development of completely new methods such as gene cloning which are only now becoming available. These require much more refinement before they will allow the reliable immortalization of

the humoral immune response for both detailed study and the production of human monoclonal antibodies.

The results presented earlier in this work suggest that there is a mature humoral immune response in many patients with breast cancer. Reliable immortalization techniques would allow the specificity of this response to be analysed and the tumour antigen to be identified and might eventually lead to the development of clinically useful human monoclonal antibodies.

CHAPTER 5: CONCLUSIONS

EVIDENCE OF AN IMMUNE RESPONSE IN BREAST CANCER PATIENTS

From the results presented in chapter 2, there is evidence of activated T lymphocytes infiltrating the tumour, attracted by tumour antigen in combination with class I MHC complexes. In most tumours, the CD8+ suppressor/cytotoxic T cells predominate although the CD4+ helper T cells were found in significant proportions in those tumours with a heavy lymphocytic infiltrate.

Both of these phenotypes express activation markers, with more of the CD8+ T cells bearing HLA DR, suggestive of antigen recognition and presentation as well as activation, while more of the CD4+ T cells carry the receptors for IL-2 and transferrin which suggest stimulation by antigen and cell division.

The presence of HLA DR bearing T cells was found to be greater in poorly differentiated tumours of high histological grade. This relationship was more marked in the CD8+ T cell subset.

All the parameters of lymphocyte infiltration and activation correlate with the presence of the class I MHC complex on the tumour cells.

The degree of activation, although not the phenotypic proportions, also correlates with the tumour cell expression of the class II MHC complex.
These findings suggest that some form of tumour antigen is present on breast carcinoma cells, particularly in poorly differentiated tumours, and that this antigen is being presented for immune recognition by the class I and class II MHC complexes, leading to a cellular immune response within the primary tumour.

The immune stimulation also includes the axillary lymph nodes where the proportion of CD8+ T cells bearing HLA DR correlates with tumour stage. The CD8+ T cells, within the nodes, would then have direct contact with the tumour cells present in nodal metastases.
Both HLA DR and the receptor for IL-2 are found on significantly more T cells, of both phenotypes, in the lymph nodes of patients with breast cancer than in those from normal controls.
In the axillary lymph nodes, the CD4+ T cell population is greatly increased in many of the cancer patients with CD4+/CD8+ ratios as high as 16:1.
Although the proportion of B lymphocytes in the lymph nodes of breast cancer patients is similar to those of controls, the proportion of these cells bearing membrane bound IgG is much greater in the cancer patients, in

some cases accounting for up to 70% of the B cells. These findings suggest antigenic stimulation of the regional immune system with the CD4+ helper T cell population greatly increased in size and both T cell subgroups showing signs of activation in patients with breast cancer.
The high proportion of IgG committed B lymphocytes, in many of the breast cancer patients, is suggestive of a mature humoral immune response.

HLA DR and the IL-2 receptor are also carried on more of the peripheral blood T cells from the cancer patients than from the controls and again a greater proportion of the B lymphocytes were positive for surface IgG.
The phenotypic constitution was found to be similar in the two groups although there was a greater range of CD4+/CD8+ ratios and slightly fewer B lymphocytes in the blood of patients with breast cancer.

Although our results show evidence of immune stimulation and response to putative tumour antigen in many patients, further studies are required to assess the functional capacity of this response, with cytotoxicity assays of the CD8+ T cells, lymphokine secretion assays of the CD4+ T cells and antibody binding studies of the IgG secreted by the B lymphocytes.

Of particular interest are the CD4+ T cells as this population is greatly increased in many of the cancer patients. A much greater proportion of these cells than CD8+ T cells bear the IL-2 receptor which is in keeping with the finding of several groups that, in IL-2 stimulated TIL culture, it is the CD4+ T cell subset which expands. There are some reports of promising results using TIL therapy for malignant melanoma and it may be that these have been mediated by CD4+ helper T cells rather than the CD8+ suppressor/cytotoxic T cells. The mechanism by which this might have been mediated requires clarification.

The impact of the immune response on disease free and overall survival would give some indication of its effectiveness but the follow up of these patients is, as yet, too short to allow any effect to be seen or conclusions to be drawn.

THE ROLE OF THE LYMPH NODES IN THE IMMUNE RESPONSE

When the three lymphocyte sources are compared, the axillary lymph nodes are found to be the major source of CD4+ helper T cells and IgG committed B cells. Although the lymph nodes are the major quantitative

source of CD8+ T cells and a large proportion of them appear to be activated, the most relevant source of these cells is the primary tumour where they predominate.
The peripheral blood lymphocytes tend merely to reflect, to a lesser degree, the status of the axillary lymph nodes, with regard to T cell subset proportions and the IgG commitment of B cells.

At the time of definitive surgery, the immune response detected in some patients appears to be regionally based rather than centralized. This is therefore likely to be damaged by conventional treatment of the breast and axilla, with surgery or radiotherapy, while the use of adjuvant chemotherapy causes generalized immunosuppression.

It may be possible to identify patients who are mounting a loco-regional immune response, cellular or humoral, by analysing the peripheral blood lymphocytes. This would allow these patients to be selected for lymph node conservation, if clinically appropriate, and possibly even for immune enhancement or immunotherapy.

IMMORTALIZATION OF THE HUMORAL RESPONSE

Despite the failure to produce a human monoclonal antibody from breast cancer patients, the problems yet to be overcome are largely technical and only when the humoral response can be easily and reliably immortalized will it be possible to assess its effectiveness. The presence, in some patients, of such a large population of IgG bearing B Lymphocytes is encouraging and the diagnostic, therapeutic and experimental potential of human monoclonal antibodies makes the continued efforts to isolate them worthwhile.

This study therefore provides evidence of antigen recognition and a regionally based immune response in some patients with breast carcinoma. This requires further functional analysis but should perhaps be taken into account when planning the treatment of the primary tumour and may provide a basis for future immunotherapy.

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