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Flow Cytometric Analysis of Tumour-draining Lymph Nodes in Breast Cancer Patients

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The phenotype and activation status of lymphocytes from the peripheral blood and axillary lymph nodes of 40 patients with breast cancer were analysed using flow cytometry and compared with lymphocytes from the blood and lymph nodes of 7 control subjects. There was little difference in the overall proportions of T and B lymphocytes but there was a much larger population of B cells bearing surface IgG and a greater number of CD4+ helper T cells, particularly in the regional nodes, in the breast cancer patients. Many more T cells in the cancer patients were found to be carrying the HLA DR and Tac antigens. The axillary lymph nodes were the major site of B cells and CD4+ T cells whilst the primary tumour was the source of the CD8+ suppressor/cytotoxic T cells. Any immune response appeared to be largely loco-regional and may therefore destroyed by conventional surgery or radiotherapy.
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INTRODUCTION

THE DEBATE about the surgical management of patients with breast carcinoma began in the 1960s, when the use of the Halsted radical mastectomy was first challenged. The major controversy at that time was over the treatment of the axillary nodes with the suggestion that the removal of these might be detrimental to those patients who were mounting a regional immune response against their tumours [1]. The emphasis of that debate has shifted, in recent years, to the surgical management of the primary lesion and the axillary nodes are excised in the majority of patients. The effect of this on the immune system is not clear. In an attempt to clarify the contribution of the regional lymph nodes to host defences, a large multicentre trial of patients with clinical stage I breast cancer was performed [2, 3]. 40% of the group in which axillary dissection was performed proved, on histological examination, to have stage II disease and as it was a randomised trial it can be assumed that a similar proportion of those undergoing simple mastectomy also had microscopic nodal metastases. While there was no overall survival difference between the two groups it was notable that only 15% of the simple mastectomy group ever presented with clinical disease in the axillary nodes while the other 25% remained clinically well after more than 10 years of follow up. This suggests that some patients remain well in the presence of occult disease and it is possible that this is due to some host antitumour immune response.

The development of monoclonal antibodies to markers on the cell membrane allows identification of the cell phenotype and activation status of the lymphocytes within any population. While these antibodies have been applied by many groups to the immunohistochemical study of the tumour infiltrating lymphocytes (TILs) [4–6] few studies have yet applied flow cytometry to this analysis [7, 8] and only two studies of the axillary lymph node lymphocytes have been performed using this method.

Morton *et al.* [9], comparing the peripheral blood and lymph node lymphocytes, found an increase in the proportion of B cells and CD4+ helper T cells in the nodal population and a greater proportion of cells bearing HLA DR. As this study included no control subjects, it was not possible to tell whether these differences related to the presence of breast carcinoma or were simply due to the lymphocyte source.

Mantovani *et al.* [10], comparing the lymph node lymphocytes (LNLs) of breast cancer patients with those from normal controls, found no major differences in the phenotypic proportions but did find a larger CD4+ T cell population in nodes invaded by cancer. The latter also studied the HLA DR expression and found no significant difference between patients with breast cancer and normal subjects.

No studies have compared the expression of interleukin 2 or transferrin receptors nor the proportion of IgG bearing B cells

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in the lymph nodes of breast cancer patients and control subjects. Pattanapanyasat *et al.* [11] used flow cytometry to compare the peripheral blood lymphocytes (PBLs) of breast cancer patients and control subjects and found an increase in both the CD8+ T cell population and the proportion of cells bearing HLA DR. Mantovani *et al.* [10] also studied PBLs and while they found no phenotypic alterations they did find an increase in the proportion of cells bearing HLA DR.

In this study we compared the peripheral blood and lymph node lymphocytes of patients with breast cancer with those from normal subjects, with regard to phenotypic proportions and activation status, to assess the possibility of an immune response within the breast cancer patients that might be directed against their tumours. We also compared lymphocytes from the primary tumour, the axillary lymph nodes and the peripheral blood within the same patients to assess the contribution of the regional lymph nodes to any immune response.

MATERIALS AND METHODS

Preparation of lymphocyte samples

Lymph node samples and 10 ml of peripheral venous blood were collected from 40 patients at the time of definitive surgery for breast cancer which included excision sampling of the ipsilateral axillary lymph nodes. Samples of the primary tumour were also obtained from 25 of these patients in whom the tumour was large enough. Half of each lymph node was sent for routine histopathology to allow accurate staging of the disease and the bulk of each tumour was sent for routine histological examination and oestrogen receptor assay. None of the patients had received any preoperative antitumour therapy.

Lymph nodes from around the iliac vessels, along with 10 ml of peripheral blood, were also collected from 2 cadaveric kidney donors and 5 patients undergoing aortic bifurcation graft surgery to act as control samples. None of these patients had any evidence of malignant disease, auto-immunity or immunosuppression.

The tumour samples were processed as described previously [8, 12]. The lymph node cells were also spilled by mechanical disaggregation and the lymphocytes were harvested from both this suspension and the peripheral blood sample using density gradient separation over Ficoll Hypaque (Pharmacia). The lymphocytes were stored in liquid nitrogen until they could be analysed by flow cytometry. Preparation was carried out as soon as possible after receipt of the samples to minimise turnover of the membrane receptors.

Flow cytometry

The flow cytometer used in this study was the FACScan Analyser (Becton Dickinson, Oxford) which uses a 15 mW argon ion laser to excite fluorochromes within the red to green waveband.

The size and granularity of cells affect the forward and side scatter of laser light and this allows differentiation between lymphocytes, monocytes and neutrophils.

Surface markers

In this study data was collected only from live lymphocytes and these were phenotyped as B cells, CD8+ suppressor/cytotoxic T cells or CD4+ helper T cells.

Using dual immunofluorescence, each phenotypic subgroup was analysed to see what proportion were carrying the activation markers HLA DR, the receptors for interleukin 2 (IL-2) and transferrin and, in the case of the B cells, surface immunoglobulin G.

Table 1. Monoclonal antibodies used in this study

Antibody 1	Antibody 2	Predominant reactivity
IgG1-FITC	IgG2a-PE	Control
Leucogate Anti-CD45-FITC	Anti-CD14-PE	Differential staining of leukocyte subpopulations (lymphocytes, monocytes, neutrophils)
Anti-Leu4-FITC	Anti-Leu12-PE	T-lymphocytes, B-lymphocytes
Anti-Leu 3a-PE	HLA DR-FITC	Activated CD4+ helper T cells
Anti-Leu 3a-PE	Anti-CD25-FITC	IL2-receptor on CD4+ helper T cells
Anti-Leu 2a-PE	HLA DR-FITC	Activated CD8+ cytotoxic T cells
Anti-Leu 2a-PE	Anti-CD25-FITC	IL 2-receptor on CD8+ cytotoxic T cells
Anti-Leu 12-PE	mouse anti-human Ig-FITC (γ chain)	surface IgG-expressing CD19+ B cells

FITC = fluorescein isothiocyanate.

HLA DR is the predominant class II antigen of the major histocompatibility complex. It is a marker of T cell activation and is associated with antigen recognition [13, 14].

Tac, the 55 kD component of the interleukin 2 receptor, is increased on the surface of lymphocytes which have been stimulated by antigen and in the presence of interleukin 2 [15].

The transferrin receptor expression increases on the surface of dividing cells [16] because of the need for intracellular iron by the enzyme ribonucleotide reductase which supplies deoxyribonucleotides for DNA synthesis.

Immunoglobulin G is produced by B cells mounting a mature or secondary immune response against an antigen and was therefore used as the indicator of a humoral response in the patients studied. The antibodies used to stain these markers were as shown in Table 1.

Staining

The basic method of cell staining was the same regardless of the lymphocyte source. The cell samples were quickly thawed to prevent damage by ice crystal formation and washed twice in filtered phosphate buffered saline (PBS). The cells were then resuspended at a density of 2×10^7/ml and 50 μl of this was aliquoted into each of 14 flow cytometry test tubes (Falcon 2052) and the fluorescent monoclonal antibodies added. The tubes were incubated for 20 min in the dark, to prevent bleaching of the PE and on ice, in the presence of 0.02% sodium azide, to prevent capping and internalisation of the antibody–antigen complexes.

After incubation the cells were washed and resuspended in filtered PBS. Propidium iodide was added, to a final concentration of 2 μg/ml, to allow the identification and exclusion of dead cells from the analysis. The samples were then run on the cytometer, setting the collection gates to exclude dead cells, monocytes and neutrophils, and accumulate data only on live lymphocytes.

Analysis

Leucogate, an antibody preparation differentially staining lymphocytes, monocytes and neutrophils was used to check the efficiency of the lymphocyte gate. The relative proportions of the phenotypic subsets were measured using four quadrant analysis and these were expressed as a percentage of the total number of cells. The activation markers were studied by isolating the phenotype of interest and then using histogram analysis of each activation marker. These results were expressed as a percentage of that phenotype bearing the activation marker.

An irrelevant control antibody was used to set the analysis gates so as to exclude non-specific binding.

Statistics

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 the lymphocytes from the breast cancer patients and also to compare lymphocytes from different sources within each patient. Correlation of the lymphocyte status in different tissues and with prognostic factors, such as tumour stage, grade or the presence of oestrogen receptors, was performed using the Spearman Rank correlation test.

RESULTS

There was little difference in the number of lymphocytes in the peripheral blood of control subjects or breast cancer patients with each 10 ml sample yielding approximately 1.5×10^7 lymphocytes on average.

The nodes collected from the breast cancer patients, however, were often larger and rather engorged when compared with those from the control subjects and yielded an average of 4×10^7 lymphocytes from each lymph node compared with less than 10^7 from the small pale control nodes.

Cell phenotypes

There was no significant difference in the overall proportion of T or B cells in the lymph nodes of the two groups although the B cell population in the peripheral blood of the patients was slightly smaller than that of the controls ($P < 0.01$).

The regional lymph nodes appeared to be the major site of the B cell population which made up 35% of the lymph node lymphocytes while accounting for only 13% of the peripheral blood lymphocytes and less than 7% of the tumour infiltrating lymphocytes ($P < 0.0001$) (Fig. 1a).

Subdivision of the T lymphocytes into CD4+ helper T cells and CD8+ suppressor/cytotoxic T cells revealed much greater variation of the size of these populations among the breast cancer patients than the controls along with an overall increase in the size of the CD4+ T cell population with a mean of 49% in the lymph nodes of the cancer patients compared with 33% in the controls ($P = 0.003$) (Fig. 1b).

The variation in the T cell subsets led to a broader range of CD4+/CD8+ ratios in the cancer patients. While the mean CD4+/CD8+ ratio in peripheral blood was 1.7, for both groups, the range in controls was from 1.07 to 2.44 while that in the cancer patients varied from 0.53 to 5.0.

In the lymph nodes the average control CD4+/CD8+ ratio was 2.0 with all results lying within a tight range while the average ratio in the breast cancer patients was 4.86 with results ranging from 1.22 to 16.25 ($P = 0.02$) (Fig. 2a). This is in contrast to the tumour where the mean CD4+/CD8+ ratio of the TILs has previously been shown to be 0.8 with the CD8+ cells predominating [8].

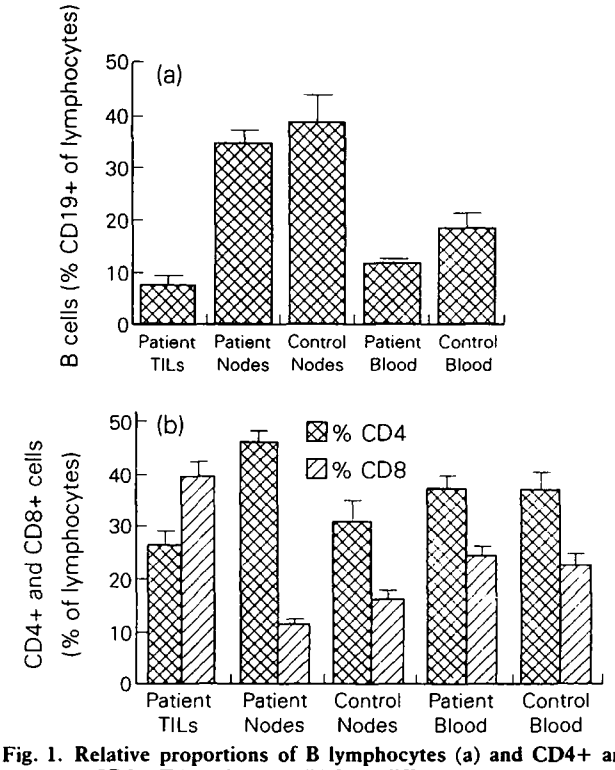


Fig. 1. Relative proportions of B lymphocytes (a) and CD4+ and CD8+ T lymphocytes (b) from different sources.

In the breast cancer patients, there was a strong correlation between the CD4+/CD8+ ratios of blood and lymph node ($P < 0.001$) (Fig. 2b) which was largely due to a strong correlation in the size of the CD4+ helper T cell populations between the two sources ($P < 0.001$). The lymph nodes were the major source of these cells which accounted for 49% of the lymph node lymphocytes and 39% of the blood lymphocytes ($P < 0.03$) (Fig. 1b).

Activation markers

HLA DR. All patient samples contained some HLA DR positive T cells but the percentage of positive cells showed wide variation. HLA DR was borne by many more T cells in the peripheral blood and regional lymph nodes of the cancer patients than in the control subjects. 36% of CD8+ T cells in the peripheral blood of the cancer patients had this marker compared with 14% of the normal CD8+ T cells ($P < 0.0001$) while in the lymph nodes 49% of the CD8+ T cells from the patients and only 18% of those from control nodes bore this marker ($P < 0.0001$) (Fig. 3a).

This increase was also seen among the CD4+ helper T cells where HLA DR was found on 19% of the CD4+ T cells in the peripheral blood of patients and only 8% in the controls ($P < 0.0001$).

In the nodes 38% of the CD4+ T cells from the patients were positive for this marker compared with 12% from the controls ($P < 0.0001$) (Fig. 3b).

The TILs contained the greatest proportion of HLA DR + T cells, being it present on up to 90% of the CD8+ cells in some cases, with an average of 53% of the CD8+ and an average of 46% of the CD4+ T cells bearing this marker. There was no significant difference between the tumour and lymph node lymphocytes but an overall smaller percentage of peripheral

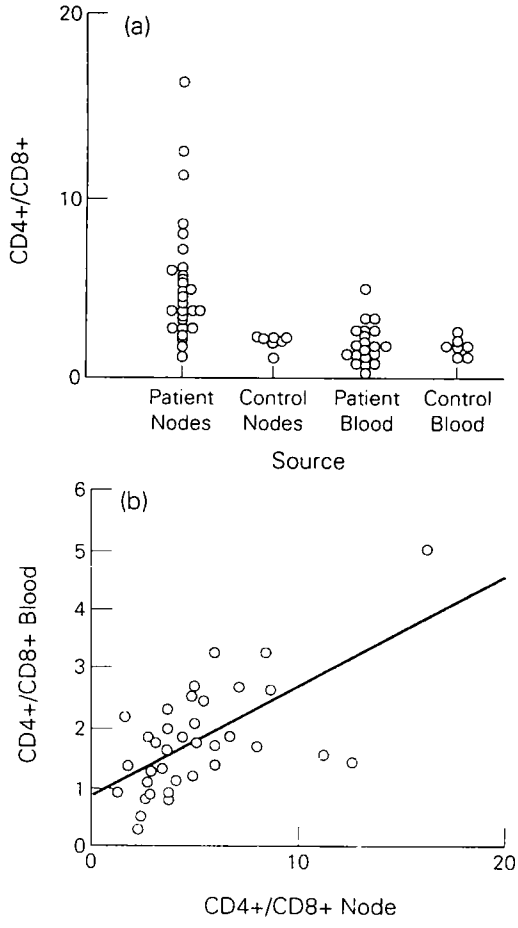


Fig. 2. (a) Distribution of CD4+/CD8+ ratios in nodes and blood of patients and controls. (b) The relationship between CD4+/CD8+ ratios in nodes and blood from patients.

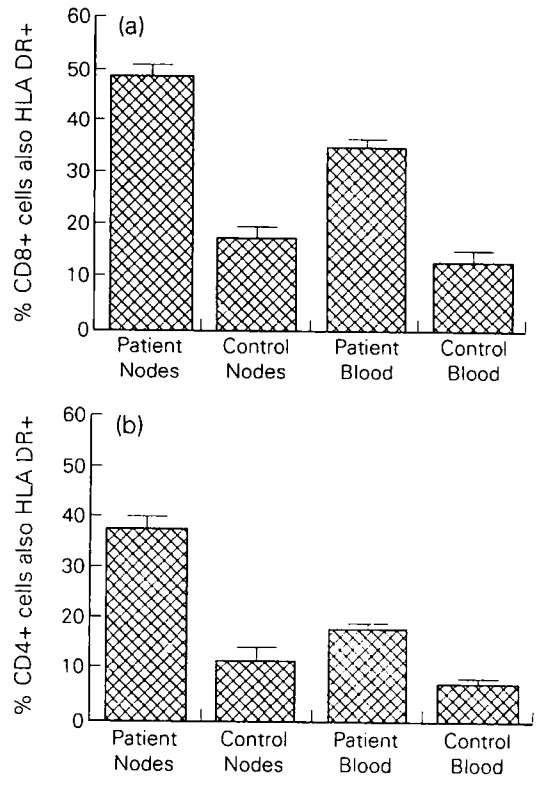


Fig. 3. (a) Proportions of CD8+ cells positive for HLA DR in patients and controls. (b) Proportions of CD4+ cells positive for HLA DR in patients and controls.

blood T cells carried this marker, it being on 36% of the CD8+ and 19% of the CD4+ T cells ($P < 0.001$).

In the primary lesion, 21 of the 25 tumours had sufficient lymphocytes for HLA DR to be measured on the T cell subsets and the expression of HLA DR on the CD8+ T cells was found to correlate with tumour grade, increasing in poorly differentiated tumours ($P = 0.003$) (Fig. 4a). In the 22 available axillary nodes from the same patient group, HLA DR expression on the CD8+ T cells correlated with tumour stage, increasing in patients with nodal metastases ($P = 0.02$) (Fig. 4b).

Interleukin 2 receptor (Tac). This receptor also was found on more T lymphocytes from both the peripheral blood and axillary lymph nodes of the breast cancer patients compared with the control subjects while there was no difference in the number of B lymphocytes carrying this marker between the two groups. Tac was found on 15% of the peripheral blood CD8+ T cells in the cancer patients compared with only 9% in the controls ($P = 0.02$) and the lymph node CD8+ T cells were similar with 16% of those from cancer patients bearing Tac while it was present on only 8% of those from control subjects ($P = 0.005$).

Tac was found on 31% of the CD4+ T cells in the peripheral blood of patients compared with 20% in the controls ($P = 0.006$) and while 23% of the CD4+ T cells in the axillary lymph nodes of the cancer patients carried Tac, only 14% of the CD4+ T cells in the control nodes did so ($P = 0.006$) (Fig. 5).

As had been noted in our previous study of the tumour infiltrating lymphocytes, this marker was virtually always present on more CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells regardless of the patient or lymphocyte source ($P < 0.001$) (Fig. 5).

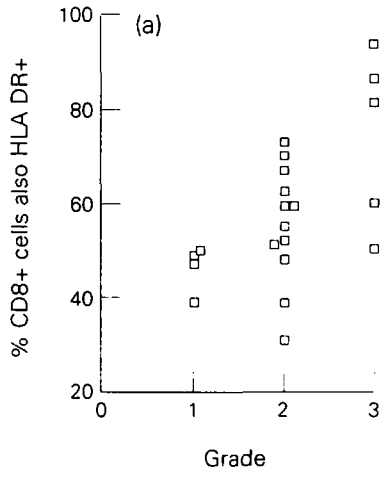
There was no correlation between the expression of this receptor and tumour grade, stage or oestrogen receptor status.

Transferrin receptor. There was no significant difference in the expression of this receptor on the peripheral blood or lymph node lymphocytes from patients with breast cancer and normal controls or any pattern relating to tumour stage or the other prognostic indicators. The greatest expression was on the CD4+ helper T cells infiltrating the tumour where it was present on an average of 48% of the cells ($P < 0.05$).

Immunoglobulin G. There was found to be a greater B cell population carrying surface IgG in the peripheral blood of the cancer patients (18%) than in the control group (7%) ($P < 0.005$) and this was also the case in the lymph nodes where 31% of the B cells in the patients compared with only 17% of the control B cells expressed surface IgG ($P < 0.03$). In both cases there was much greater variation among the cancer patients with some having more than 50% of their B cells committed to IgG (Fig. 6a).

The axillary lymph nodes were found to be the major source of these cells which accounted for 31% of the large B cell population compared with 18% of that in peripheral blood ($P < 0.001$) (Fig. 6a).

There was some correlation between the size of the IgG bearing B cell population and the overall B cell population within the axillary nodes of the breast cancer patients and a



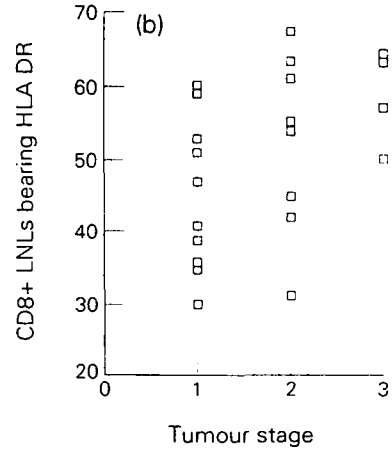


Fig. 4. (a) The relationship between HLA DR positive cells and tumour grade in the CD8+ population from the tumour infiltrating lymphocytes. (b) The relationship between HLA DR positive cells and tumour stage in the CD8+ population from the lymph node lymphocytes of patients from the same group (the significance is upheld ($P = 0.02$) when a further 11 patients for whom primary tumour was not available are added to the study).

fairly strong correlation between the size of this population in the peripheral blood and lymph nodes of the cancer patients ($P < 0.001$) (Fig. 6b). There was, however, no correlation with any features of the primary tumour itself.

DISCUSSION

From these results it is clear that there are major alterations of both the phenotypic proportions and activation status of the lymphocytes, particularly in the axillary lymph nodes, of patients with breast cancer. While there was no alteration in the lymphocyte yield from the peripheral blood of these patients, the grossly engorged appearance of some lymph nodes was associated with greatly increased lymphocyte yields. This increase in size has been noted previously [17, 18] and contrasts sharply with the small pale control nodes.

Cell phenotypes

No difference was found in the proportion of T or B lymphocytes in the axillary lymph nodes of breast cancer patients and

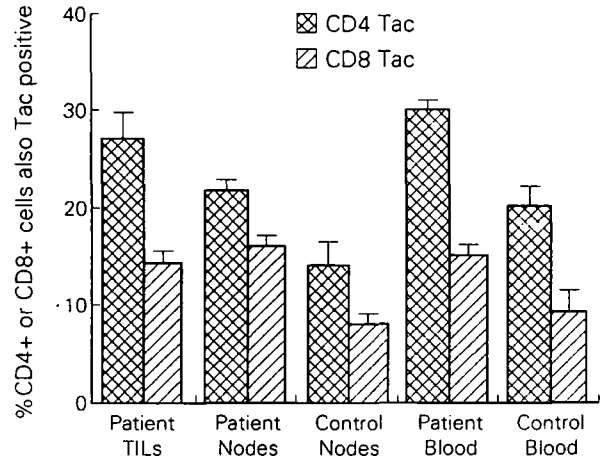


Fig. 5. Levels of Tac (the 55 kD Il-2 receptor) on T lymphocytes of patients and controls.

normal controls and although there was a slight decrease in the number of B cells in the peripheral blood of these patients, there was little alteration in the T cell population. These findings are contrary to those of Eremin *et al.* [18] who found an increase in the lymph node B cell population and several groups who found a decrease in the number of circulating T cells [19–21] with the

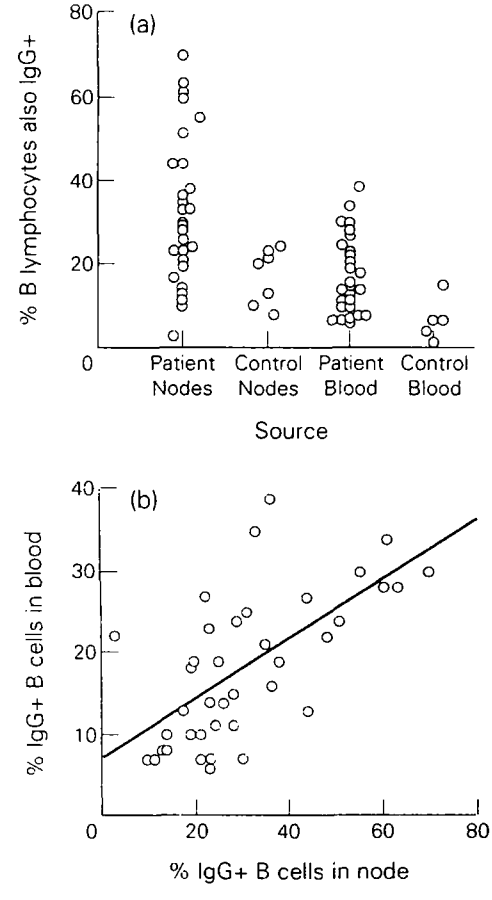


Fig. 6. (a) Percentages of B lymphocytes positively stained for surface IgG in patients and controls. (b) Relationship between B lymphocytes positive for surface IgG in patient lymph nodes and peripheral blood.

last group suggesting a compensatory rise in the B cell numbers. Some groups also noted an increase in the number of peripheral blood B cells in patients with stage II disease [9, 18, 22] and while this trend was present among our patients it was very weak and not statistically significant.

The further subdivision of the T lymphocytes into CD8+ suppressor/cytotoxic T cells and CD4+ helper T cells showed more marked differences between the two groups. The average CD4+/CD8+ ratios and T cell subsets of peripheral blood were similar between the two groups but there was much greater variation and a wider range of values in the lymph nodes of the breast cancer patients while those of the normal controls fell within a fairly narrow range possibly representing "normal" values.

The T cell subsets of peripheral blood have been studied by two groups using flow cytometry [10, 11]. Like ourselves, Mantovani *et al.* [10] found no phenotypic alteration in the breast cancer patients but Pattanapanyasat *et al.* [11] found a greater CD8+ T cell population in these patients. Several groups [23–25] used fluorescent microscopy to study the PBLs of patients with breast cancer undergoing adjuvant chemotherapy or radiotherapy. While they found the initial CD4+/CD8+ ratios to be approximately 2:1, they found a marked fall in the ratio, due to a selective loss of CD4+ helper T cells, with ensuing immunosuppression. The initial samples in these studies did not show as much variation as those in the present study but this may be due to the fact that they were postoperative samples and surgery itself may have some immunosuppressive effect [26]. This, along with the wide variation in cancer patients, might explain why Pattanapanyasat *et al.* [11] found CD8+ T cells to predominate in the peripheral blood of breast cancer patients. An even greater range of ratios was seen in the axillary lymph nodes and while the CD4+/CD8+ ratios, in the control group, clustered around the average of 2:1 those in the cancer group ranged up to 16:1 with the average ratio being almost 5:1 due to a widespread increase in the size of the CD4+ helper T cell population in many patients. The other study which has compared the T cell phenotypic subset proportions in the regional lymph nodes of patients with breast cancer and normal subjects found no phenotypic differences between the two groups, although they did note a greater CD4+ T cell population in nodes which contained metastatic deposits [10]. This study was very small, containing <10 patients, and the source of normal lymph nodes is not given. Morton *et al.* [9], who compared LNLs with PBLs, also noted the large CD4+ T cell population in the axillary nodes of breast cancer patients but lacking normal controls, could not clarify whether this was due to the presence of malignant disease or merely due to the source of the lymphocytes. The results of this study would suggest it is the former although the axillary lymph nodes certainly appear to be the major site for the CD4+ helper T cells. In the present study the CD4+/CD8+ ratio of peripheral blood was found to reflect that in the axillary lymph nodes and so analysis of a blood sample might give some indication of the T cell subset proportions in the nodes.

Activation markers

HLA DR. There was a great increase in the number of T cells, of both phenotypes, bearing HLA DR in the peripheral blood and axillary lymph nodes of the breast cancer patients as compared with the control group. Its expression on the CD8+ T cells in the primary lesion was seen to correlate with tumour grade while on the CD8+ T cells in the axillary nodes, it correlated with disease stage. As HLA DR is the predominant antigen of the class II major histocompatibility complex and therefore plays an important role in antigen recognition it is likely that tumour antigen on the cells of poorly differentiated high grade tumours is being recognised by the CD8+ suppressor/cytotoxic T cells. Morton *et al.* [9] also found a high proportion of the axillary lymph node T cells to bear HLA DR in patients with stage II disease but Mantovani *et al.* [10] found no such correlation. As in the present study, Pattanapanyasat *et al.* [11] and Mantovani *et al.* [10] found HLA DR on a greater proportion of the peripheral blood T cells of breast cancer patients than controls. Several studies have found a similar degree of HLA DR expression in the presence of a renal transplant supporting its relationship with chronic antigenic stimulation [27, 28].

Interleukin 2 receptor. While the expression of this receptor also remained unaltered in the B lymphocyte population it was present on a greater proportion of T lymphocytes in both the peripheral blood and the axillary nodes of patients with breast cancer compared to normal subjects. The presence of Tac, the 55 kD component of the interleukin 2 receptor suggests that these cells have been stimulated by antigen and are being maintained by the presence of interleukin 2 in the cell environment. There are no other studies of this marker's expression on LNLs while the only other study of PBLs shows no difference between normal controls and patients with breast cancer [11]. As with the TILs [8], Tac was found to be present on more CD4+ helper T cells than CD8+ suppressor/cytotoxic T cells regardless of the lymphocyte source studied.

Immunoglobulin G. In this study more than twice as many of the B lymphocytes in the peripheral blood and regional lymph nodes of the breast cancer patients were found to carry surface IgG compared with the controls which is suggestive of a mature humoral immune response. Richters and Kaspersky [29] were the first to study the surface immunoglobulins on the lymph node B cells of breast cancer patients and they found an average IgG committment of 22%. Eremin *et al.* [18] found similar proportions of IgG bearing B cells in the lymph nodes of control subjects and breast cancer patients as was found in this present study. This IgG response appears to be largely situated in the regional lymph nodes as the tumour and blood have much smaller B cell populations. The strong correlation between the proportion of B cells bearing surface IgG in the axillary lymph nodes and in the peripheral blood might again allow analysis of a blood sample to indicate the level of IgG response within the nodes.

Further study of the specificity of this IgG is required but some initial immunohistochemical analysis of autologous tumour with short term culture supernatant from these B cells showed promising staining. It was not technically possible to immortalise a monoclonal antibody from these IgG producing B cells which would have allowed detailed study of their antigen binding.

Conclusions

There is evidence from these results to suggest recognition of tumour antigen particularly by the CD8+ suppressor/cytotoxic T lymphocytes infiltrating the tumour or within lymph nodes containing metastatic deposits. These cells are the major constituent of the tumour infiltrate and as their role is in cell to cell direct killing, this gives them good access. After destruction of the tumour cells, it is possible that some of the antigenic material

from the tumour cells is being presented, within the groove of the class II major histocompatibility complex (HLA DR), to the CD4+ helper T cells which then coordinate the rest of the immune system and its response.

Although activated CD4+ T cells are found within the tumour itself, the main site of these cells appears to be the axillary lymph nodes. The helper T cell population is greatly increased in the lymph nodes of breast cancer patients compared with normal subjects. The greater expression of the interleukin 2 receptor on the surface of these cells is likely to make them more susceptible to stimulation by exogenous interleukin 2 and may have some therapeutic potential.

The axillary lymph nodes are also the major site of the B lymphocytes bearing surface IgG suggestive of a mature humoral immune response. Further studies of the functional capacity of the lymphocytic phenotypes are needed to assess the specificity of the IgG produced by the B lymphocytes, the cell killing capacity of the CD8+ suppressor/cytotoxic T lymphocytes and the cytokine secretion and cell communication abilities of the CD4+ helper T lymphocytes. This latter cell type would appear to be of particular importance and better understanding of their function might allow development of new immunotherapeutic approaches.

Although the peripheral blood appears to reflect the situation within the regional lymph nodes to some extent, it shows much less phenotypic alteration and cell activation suggesting that at the time of definitive surgical treatment the immune response, if present, is still loco-regionally based.

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