Eur J Cancer Clin Oncol, Vol. 22, No. 2, pp. 219–223, 1986.
Printed in Great Britain.

0277–5379/86$3.00+0.00
Pergamon Press Ltd.

Sinus Histiocytes in Axillary Lymph Nodes from Patients with Breast Cancer: Macrophage Characteristics and Activation Level*

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Abstract—*Cell suspensions from 16 tumour-free axillary lymph nodes from breast cancer patients were prepared, using collagenase digestion to free the sinus histiocytes from the fibrous stroma of the nodes. The histiocytic cells so obtained were then characterized using four surface markers: Fc(IgG) receptors, C_3 receptors, DR antigen and a macrophage-associated antigen (defined by the monoclonal antibody VEP-7). In addition phagocytosis was assessed using IgG-coated red cells, and both lysozyme and α-1-antitrypsin were localized by means of immunoperoxidase staining. The results demonstrated that the majority of sinus histiocytes carried surface macrophage markers, but that a minority displayed phagocytosis and the presence of lysozyme or α-1-antitrypsin.*

INTRODUCTION

SINUS histiocytosis is a histological feature which is seen in tumour-free lymph nodes draining human breast cancer, and its presence has been correlated with a favourable prognosis[1–4]. It consists of dilated intranodal sinuses filled with large cells having irregular, vesicular nuclei and eosinophilic cytoplasm, and the term 'histiocytosis' implies that these cells morphologically resemble tissue macrophages. However, despite a large volume of work on the significance of histological sinus histiocytosis[1–2], the precise nature of these cells and their functional or immunological characteristics are poorly defined.

The present study was therefore designed to investigate the possible macrophage nature of sinus histiocytes, by extracting them from lymph nodes and by using rosetting techniques to detect Fc(IgG) and C_3 receptors, DR antigen and a macrophage-associated antigen defined by the monoclonal antibody VEP–7[7]. Further characterization was obtained by assessing phagocytosis of IgG-coated erythrocytes and the presence of cellular lysozyme and α-1-antitrypsin (A1AT) detected by immunoperoxidase techniques.

*This work was supported by a grant from the Cancer Research Campaign (U.K.).
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Accepted 3 September 1985.

MATERIALS AND METHODS

Collection and preparation of lymph nodes

Twenty individual axillary lymph nodes, each from a different patient, were obtained during mastectomy for invasive breast cancer. As soon as the axillary nodes were removed, a macroscopically tumour-free node was dissected out of the axillary fat under sterile conditions in the operating theatre and placed immediately into tissue culture medium (TCM). TCM consisted of RPMI 1640 with 10% heat-inactivated foetal calf serum (FCS), streptomycin 100mg/ml, penicillin G 100,000 i.u./ml, sodium bicarbonate 0.7 g/l and 25mM HEPES buffer.

The node was then transported to the laboratory, where a section was taken for histology. Multiple puncture and distension of the node was carried out using a 23G hypodermic needle and a syringe containing TCM. Lymphocytes were readily expelled from the node into the surrounding medium. The remaining stroma was then mechanically disaggregated using a scalpel, and incubated in collagenase (Sigma, type I, 300 u/ml) for 12 hr at 37°C with continuous gentle rotation to isolate the retained histiocytes.

The use of collagenase was found to be necessary, as multiple puncture and mechanical disaggregation alone yielded very few histiocytic cells. Microscopic examination of residual stroma after mechanical extraction of cells, however, demons-

trated many histiocytic cells adhering to the strands of fibrous tissue. The collagenase treatment of the stroma described above produced an optimum percentage (9–50%) of large (15–40 μm) histiocyte-like cells as determined by phase-contrast microscopy and on cytocentrifuge preparations.

Following enzymatic treatment, the container was shaken vigorously, and the contents filtered through sterile gauze layers and washed six times in TCM to remove residual debris and tissue fragments. The cells were then resuspended in TCM at 2×10^6/ml, and cell numbers and viability were assessed using phase contrast microscopy.

Detection of cell surface markers

Four surface markers were studied: the receptors for the Fc portion of IgG and for C_3. HLA-DR antigen, identified by the mouse monoclonal antibody BT 2/9, and a macrophage-associated antigen defined by the mouse monoclonal antibody VEP-7. Fc(IgG) receptors were detected by means of the EA-rosetting reaction. Briefly, ox erythrocytes (E), were incubated with a subagglutinating but optimal dose of rabbit IgG anti-ox erythrocyte antiserum (A) (1:100 in PBS) for 45 min at room temperature (RT)[8]. The sensitized EA indicator cells (2% suspension in TCM) were mixed with equal volumes of the lymph node cell suspensions (2×10^6 cells/ml) and spun down. The pellet was allowed to stand at 4°C for 30 min and then resuspended by slow, continuous rotation for 1 min. C_3 receptors were demonstrated using the EAC-rosetting reaction. Ox erythrocytes (E) were incubated with a subagglutinating but optimal dose of rabbit IgM anti-ox erythrocyte antibody (A) (1:40 in PBS) for 15 min at 4°C and then with an equal volume of C_5-deficient mouse serum (1:10 in complement fixation test diluent) for 15 min at 37°C [8]. The sensitised EAC indicators were then used in rosetting reactions as described above. Appropriate controls (heat-inactivated complement, EA-IgM) were set up and found to be negative. Fc(IgM) activity was absent in TCM[9]. The HLA-DR antigen and the macrophage-associated antigen were detected by the direct antiglobulin rosetting reaction (DARR), as detailed previously[10]. In this assay, the appropriate antibody was coupled, using 0.02% chromic chloride, to trypsin-treated sheep erythrocytes. The latter were slowly rotated at RT for 60 min, washed in PBS, made up to a 1% suspension in PBS and stored at 4°C. The efficacy of coupling was checked by a reverse passive haemagglutination test using rabbit anti-mouse immunoglobulin antibody (Dako). The cells to be tested (2×10^6/ml in PBS) were mixed with an equal volume of indicator cells, spun down and allowed to stand at 4°C for 30 min. Resuspension was carried out as above.

Cytocentrifuge preparations of the rosetted cell suspensions were then made, fixed in absolute ethanol for 5 min and stained using haematoxylin. The percentage of histiocytic (non-lymphocytic) cells forming rosettes (>5 erythrocytes attached to cell) was then assessed by counting 100 cells on each slide.

In order to assess the effect of collagenase in the preparative technique used to isolate the sinus histiocytes, 1×10^7 mononuclear cells isolated on Ficoll-Hypaque from the blood of 10 healthy volunteers were incubated with 15 ml of collagenase for 12 hr, washed six times in TCM and resuspended. Cell numbers and viability were assessed, and the monocyte surface receptor-markers were then determined as outlined above on treated and non-treated cells.

Assessment of phagocytosis

Lymph node cell suspensions were mixed with sensitized EA indicator cells as described above, and incubated in a pellet at 37°C for exactly 2 hr in order to allow phagocytosis of the IgG-coated indicator cells. The cells were then resuspended by rotation, and cytocentrifuge preparations were made as described above. The percentage of rosetting cells which had phagocytosed at least one red cell was then estimated by counting 100 cells on each slide.

Detection of lysozyme and A1AT

In the cytocentrifuge preparations of the rosetted cell suspensions, prepared as described above, lysozyme and A1AT were detected using the peroxidase–antiperoxidase method[11]. Rabbit anti-human lysozyme and A1AT antibodies (Dako), both diluted 1:400 in 5 ml of Tris-buffered saline containing 2% normal swine serum, were employed as the primary antibodies. These were followed by swine anti-rabbit immunoglobulin (Dako) and then by rabbit immunoglobulin against horseradish peroxidase conjugated with peroxidase (Dako). A reddish-brown colour, indicating the presence of the peroxidase, was produced by 3-amino-9-ethylcarbazole diluted 1:10 with acetate buffer and one drop of 30 vol H_2O_2. The slides were incubated in this reagent for 5 min at RT and counterstained using haematoxylin. On each of the immunoperoxidase-stained cytocentrifuge preparations the percentage of positively staining histiocytic cells was estimated by counting 100 cells.

Histological assessment

To investigate the relationship between the large, non-lymphoid cells and histological sinus

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

In addition, the paraffin sections were stained for lysozyme and A1AT using the immunoperoxidase technique described above. The percentages of sinus histiocytes which were positive for these two markers were then estimated histologically.

RESULTS

Of the 20 lymph nodes studied, subsequent histological scrutiny revealed tumour deposits in three, and no sinus histiocytosis in one. These nodes were therefore excluded from the study, leaving 16 evaluable specimens.

Surface markers

The majority of the large, histiocytic cells from the nodes expressed Fc(IgG) receptors (mean = 80.5%), HLA-DR antigen (mean = 94.8%) and the VEP-7 macrophage-associated antigen (mean = 95.6%) — see Table 1. Fewer cells carried detectable C_3 receptors, but this was probably due to the collagenase treatment (see on).

Phagocytosis

Phagocytosis of IgG-coated red cells was seen in a variable but generally small proportion (mean = 31.5%) of the large cells from the nodal cell suspensions — see Table 1.

Lysozyme and A1AT

The presence of lysozyme (mean = 14.2%) and A1AT (mean = 13.6%) was detected in only a minority of the large nodal cells — see Table 1.

Table 1

Marker	Percentage of large cells displaying marker*
Fc(IgG) receptor	80.5 ± 2.9 (60–96)
C_3 receptor	59.7 ± 2.8 (49–76)
DR antigen	94.8 ± 0.6 (92–97)
VEP-7 antigen	95.6 ± 0.9 (91–99)
Phagocytosis	31.5 ± 5.5 (8–60)
Lysozyme	14.2 ± 1.7 (7–24)
A1AT	13.6 ± 6.7 (2–25)

*Values expressed as mean ± standard error (range).

The cells which did stain positively invariably carried Fc(IgG) receptors, HLA-DR antigen and VEP-7-defined antigen. Correlation with C_3 receptor expression was less definitive due to the artefact in C_3 receptor expression produced by the collagenase preparation (see below).

Histological assessment

There was a positive correlation between the percentage of large, histiocytic cells seen on the cytocentrifuge preparations of the lymph node cell suspensions, and the percentage cross-sectional area occupied by histological sinus histiocytosis. This correlation was significant at the 0.01 level using Kendall's rank correlation[12]. Furthermore, the percentages of cells staining for lysozyme and A1AT were similar in both the tissue sections and in the cell suspension preparations.

Control experiments

Preincubation of blood monocytes with collagenase did not markedly alter the expression of Fc(IgG) receptors, HLA-DR antigen or VEP-7-defined antigen. EAC-rosette formation, however, was inhibited, indicating that C_3 receptors were stripped off or altered by the collagenase preparation. The latter contained small amounts of trypsin, which is known to modify the surface receptor for C_3[13]. Similar collagenase control experiments showed that pretreatment with collagenase had no significant effect on the phagocytic ability, or on the lysozyme and A1AT content of the monocytes. Only small, non-specific cell losses occurred during incubation with collagenase.

DISCUSSION

Reactive changes in lymph nodes draining tumours has generated interest amongst histopathologists for many years. In 1898, Halstead noted that 'certain proliferations' in lymph nodes draining breast cancers were associated with a good prognosis[14] and in 1906 Schindler reported that lymph node metastases were less common in the presence of sinus cell hyperplasia[15]. It was Black and his colleagues, however, who provided the major stimulus to research in this field by their finding that sinus histiocytosis in the axillary nodes was associated with an improved 5-yr survival in patients undergoing mastectomy for invasive breast cancer[16]. This observation has been confirmed by a number of subsequent studies from Black's group[17–21] and by several independent studies[22–27] A few reports, however, have failed to detect survival advantage in breast cancer patients with sinus histiocytosis in their axillary nodes[28–30] but this discrepancy may be due to the subjective nature of the histological grading employed in the various studies. Overall, the litera-

ture supports the view that sinus histiocytosis is a favourable prognostic factor in breast cancer, albeit a difficult variable to quantify accurately and consistently.

Sinus histiocytosis has been defined as the filling of medullary sinuses by large cells with eosinophilic cytoplasm and irregular nuclei[16], but the nature of these cells has not been unequivocally established. Animal experiments have revealed that injected colloid taken up by regional lymph nodes will accumulate largely in sinusoidal cells, indicating a phagocytic function[31,32] and ultrastructural studies have demonstrated that nodal histiocytes display features characteristic of macrophages[33,34]. To the best of our knowledge, however, cell surface studies of individual cells have not been described previously, probably due to the difficulties involved in obtaining suitable cell suspensions from lymph nodes.

In the present investigation, using collagenase digestion, large cells (15–40u) released from the stroma of tumour-free lymph nodes draining human breast carcinoma have been studied, and their presence in cell suspensions correlated with the degree of histological sinus histiocytosis. The majority of these cells carried Fc(IgG) receptors, C_3 receptors, DR antigen and macrophage-associated antigen, providing evidence that they possess macrophage characteristics. However. only a minority displayed phagocytosis, and only small numbers contained lysozyme or A1AT. Our findings suggest that phenotypically sinus histiocytes resemble macrophages, but appear to be at a low level of activation, as both phagocytosis of IgG-coated red cells[35] and intracellular lysozyme[36] are associated with activation.

The role of sinus histiocytes and the significance of their association with a good prognosis in breast cancer is unclear. This particular reactive change is associated with expansion of the paracortex of the lymph node[37–39], suggesting that sinus histiocytes might be engaged in presenting antigen to T-lymphocytes. However, sinus histiocytosis does not appear to be related to T-cell percentages in the lymph node[39] or to basal thymidine uptake[40]. Recent evidence has emerged showing that sinus histiocytosis in axillary nodes can be induced by breast biopsy[41], which indicates that it may represent a non-specific reaction. The data presented in this paper would tend to support such a view, although the possibility remains that sinus histiocytosis may be a reaction to increased traffic of tumour-associated antigen.

Acknowledgements—We thank Professor A. P. M. Forrest of the University Department of Clinical Surgery for allowing us to study patients under his care, and Professor Sir Alastair Currie for access to pathological specimens. The monoclonal antibody BT2/9 was a gift from Dr H. Waldman (Cambridge) and the monoclonal antibody VEP-7 was a gift from Dr D. Kraft (Vienna).

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