

IDENTIFICATION OF MONONUCLEAR CELLS INFILTRATING BASAL CELL CARCINOMAS

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Abstract. The identification of mononuclear cells extracted from 12 basal cell carcinomas has been carried out by means of various assays such as the E rosette test, FITC anti-Ig conjugates and peroxidase conjugates viewed at an ultrastructural level. The relative quantities of B and T cells have been determined as well as the morphology of the extracted cells. It was found that the T/B ratio is close to that of a delayed-type reaction to tuberculin, suggesting a possible identical immune mechanism, and also that the morphological appearance of some tissue lymphocytes differs from that of circulating lymphocytes due to the presence of both membrane and cytoplasmic Ig.

Key words: Basal cell carcinoma; Lymphocyte; Cell extraction

Little is known about the nature of the cells infiltrating basal cell carcinomas (BCC). Mononuclear cells extracted from the tissues can be submitted to the same immunological assays as circulating cells. We have therefore performed various tests based on the membrane properties of mononuclear cells, such as the E rosette test, EAC rosette test, FITC, and peroxidase anti immunoglobulin (Ig) conjugates. Furthermore we have studied the ultrastructural morphology of the extracted cells combined with an immunocytochemical identification with Fab peroxidase conjugates. In this paper we present our results which show that the B/T ratio is almost identical with that of a delayed-type reaction and that the ultrastructural appearance of some tissue lymphocytes differs from that of circulating lymphocytes.

MATERIAL AND METHODS

Patients

The mononuclear cells were obtained by excision of basal cell tumours from 12 patients. Whenever possible,

lymphocytes from both the skin infiltrates and the peripheral blood were studied.

Isolation of circulating lymphocytes

40 ml of peripheral blood was drawn into a syringe containing 250 units of preservative-free heparin. The lymphocytes were isolated on a layer of Ficoll Metrizoate by centrifugation at 500 g for 30 min and the final concentration of cells adjusted according to the different assays.

Extraction of lymphocytes from tissues

Tissue specimens were minced with scissors and scalpel into Hanks' balanced salt solution (pH 7.2). The minced tissue and supernatant fractions were filtered through a cotton column. The cells were centrifugated at 500 g for 10 min at 4°C. Contaminating red blood cells were lysed with ammonium chloride. After washing, cells were adjusted so as to have a sufficient number of lymphocytes for the different tests. 90% of the cells excluded Trypan Blue.

Rosette formation on circulating and extracted lymphocytes

Erosettes: 0.1 ml of a 2×10^7 cells/ml suspension of lymphocytes was incubated at 37°C for 1 hour with 0.1 ml of foetal calf serum. Sheep red blood cells were added at a concentration of 10^8 per lymphocyte. The mixture was spun at 300 g for 3 minutes and incubated 12 hours at +4°C. The cells were then resuspended gently. 400 cells were counted immediately under a light microscope at a magnification of 100× and the percentage of lymphocytes that formed rosettes with more than 3 red blood cells was calculated.

EAC rosettes: one volume of a 5% concentration of sheep red blood cells was incubated with one volume of diluted 1:100 IgG rabbit anti-sheep red blood cells at 37°C for 30 min. After washing, one volume of mouse serum diluted 1/5 in Veronal buffer saline was added and incubated at 37°C for 30 min. EAC complexes were washed and adjusted to a concentration of 1×10^6 red blood cells per ml and added to a suspension of 2×10^6 lymphocytes/ml. Cells were resuspended on a whirlmixer and EAC rosettes were counted in the same way as were the E rosettes.

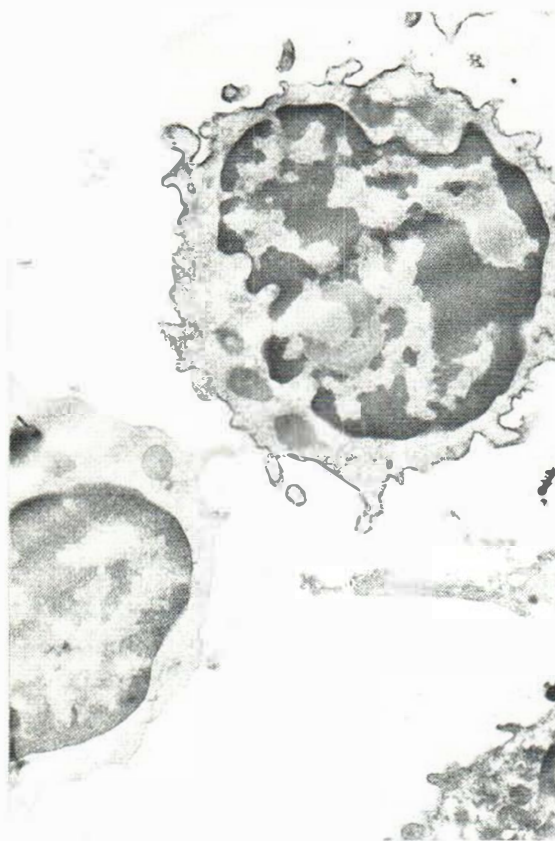


Fig. 1. Circulating lymphocytes (Fab peroxidase conjugate). Upper: B type lymphocyte with a microvillous membrane, surrounded by a thin, continuous band of staining. Lower: T type lymphocyte with a smooth membrane. Note the absence of peripheral labelling. $\times 5200$.

Immunofluorescence (IF)

If staining of lymphocyte surface Ig was done with rabbit anti-human IgG, IgM and IgA conjugated with fluorescein isothiocyanate and diluted 1/30 (Behring-Hoescht). 0.1 ml of a 2×10^7 lymphocytes per ml suspension was incubated with the FITC conjugate for 1 hour at $+4^\circ\text{C}$. After incubation, cells were washed in PBS and mounted on a glass slide in one drop of glycerol in phosphate buffer. Membrane fluorescence was examined under a Leitz fluorescence microscope (Incident light, Orthoplan) by counting 400 cells.

Electron microscopic study

Tissue specimens were fixed with 2% glutaraldehyde at $+4^\circ\text{C}$ for 3 hours. After an overnight wash in cacodylate buffer, 1 mm^3 blocks were post-fixed with 1% osmium tetroxide in sodium cacodylate buffer 0.4 M for 2 hours at $+4^\circ\text{C}$. After rinsing in water and dehydration in alcohol, the tissue fragments were soaked in epoxy resin for 3 days. After polymerisation at $+60^\circ\text{C}$ for 3 days, the blocks were cut with a Reichert ultramicrotome. One

Table 1. Percentage of subpopulations of lymphocytes in various normal lymphoid tissues and blood

ND: not done. LM: light microscopy

	E-RFC ^a %	EAC-RFC ^a %	FITC anti- Ig %	Fab perox. ^b % LM
Peripheral blood	65 \pm 7	15 \pm 5	22	10
Thymus	96	<1	<1	<1
Lymph node	46	27	33	ND
Spleen	30	34	35	44
Tonsil	42	50	42	47

^a RFC: rosette-forming cell.

^b Fab perox.: Fab peroxidase conjugate.

micron semi-thin section were mounted on a slide and stained with a mixture of methylene blue-Azur II. The ultra-sections mounted on a grid were stained with uranyl acetate/lead citrate and examined with a Philips EM 300 or Hitachi HU 12-A electron microscope.

Histological examination

The samples were fixed direct in Baker fixative and embedded in paraffin. They were then stained with hematoxylin-eosin-safran.

Immunoenzymatic methods

For examination under the light microscope the cells were washed and then spread on a glass slide for 30 min at 37°C . After a light fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and washing with

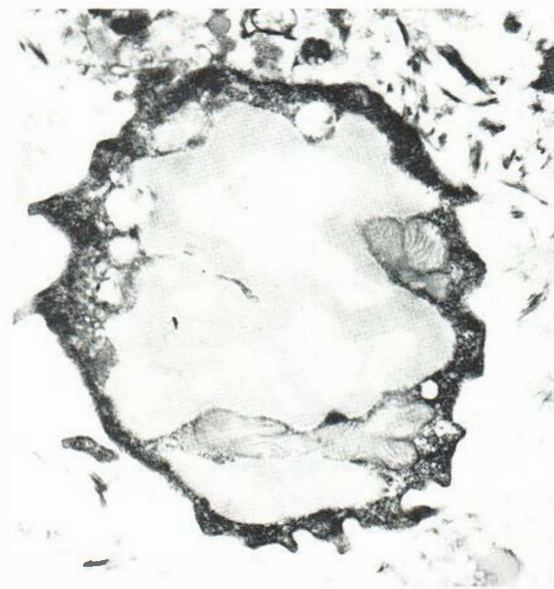


Fig. 2. Extracted lymphocytes (Fab peroxidase conjugate). Note the absence of microvilli and membrane and cytoplasmic Ig. $\times 5400$.



Fig. 3. Detail of Fig. 2. $\times 15\,000$.

PBS, the cells were then layered with Fab-peroxidase conjugate diluted 1/10 (Fab fragments of sheep Ig anti-human Ig, Institut Pasteur, Paris), for 30 min at 37°C. After washing with PBS, the cells were fixed with 2% glutaraldehyde in sodium cacodylate buffer for 15 min at room temperature. Following a wash in sodium cacodylate buffer and then in 0.2 M Tris-HCl buffer, pH 7.6, the peroxidase activity was demonstrated using Graham-Karnovsky mixture (3-3' diaminobenzidine, 5 mg per ml Tris-HCl buffer in the presence of added hydrogen peroxide). After washing, the preparations were covered with a cover-slip using glycerin as the mounting medium and examined through a $\times 100$ immersion lens. These preparations could be stored (2).

Electron microscopical examination of cell pellets

The usual fixation methods for the preparation of material for routine ultrastructural examination were followed,

with some variation in the duration of the different stages. The cells were fixed in suspension with paraformaldehyde for 20 min, then washed and put in contact with the conjugate for 3 hours at 37°C. After washing and fixation, the peroxidase activity was demonstrated by reacting with 3-3' diaminobenzidine for one hour in the dark. After washing, the cells were post-fixed for 20 min with osmium tetroxide using the standard method described above. The cells were examined in a Philips EM 300 at 40 and 60 kV without contrast.

RESULTS

Control studies of the tests were made on normal circulating lymphocytes and on normal human lymphoid tissue (foetal thymus, lymph nodes, spleen and tonsils). The quantitative results given in Table I were comparable to those given in the literature (4, 11). In BCC the percentages of circulating B and T cells are comparable to those of normal subjects.

The cells extracted from the skin were tested mainly by their capacity to form E and EAC rosettes, and by their staining by peroxidase conjugate as viewed under the electron microscope. Few of the biopsies were able to provide a sufficient number of cells to enable us to use all these different methods. The quantitative results are shown in Table II, compared with those of delayed hypersensitivity reactions (DHR). Statistical evaluation of the results allowed us to calculate the 95% confidence limits of the mean value. Cells extracted from DHR are mainly E-RFC (79%+5) with a T/B ratio of 5.3. 60% of the cells extracted from BCC form E-RFC with a T/B ratio of 4.6

In the electron microscope the immuno-peroxidase permits us to distinguish clearly two different types of lymphocytes in the blood (Fig. 1). The type B cells are rounded, and have a high nucleocytoplasmic ratio. The nucleus is round and only slightly indented, the peripheral heterochromatin is very dense, the cytoplasm contains few organelles, and the membrane was thrown into numerous villi.

Table II. Confidence limits of the mean values of E and EAC-RFC in cases of cells extracted from delayed hypersensitivity reactions (DHR) and basal cell carcinomas (BCC)

	No. of cases	E-RFC %		EAC-RFC %		E/EAC RFC
		Mean	Range	Mean	Range	
DHR	8	79 \pm 5	74-84	15 \pm 4	11-19	5.3
Basal cell carcinoma	12	60 \pm 4	55-65	13 \pm 2	11-17	4.6

The stain was dense and evenly distributed, on the cytoplasmic membrane without labelling the cytoplasm. Type T lymphocytes had the same general structure of organization of their nucleocytoplasm but had a smooth surface without any labelling by immunoperoxidase, on either the membrane or the intracytoplasm (7).

Within the tissues, some cells appear similar to circulating B and T cells but other cells differ clearly from normal B cells (Figs. 2, 3). Microvilli, normally present on circulating B lymphocytes, are absent. The Fab peroxidase labelling is present not only at the surface membrane but also at the peripheral part of the cytoplasm. These cells retain the main characteristics of lymphocytes and were closely allied to B cells by the presence of globulin on their surface membrane. The same aspect has been found with FITC conjugates and on tissue sections in electron microscopy (3). Consequently the extraction procedure does not create an artefact. We propose to call this cell tissue B lymphocyte (Bti).

DISCUSSION

Only a few studies of this type have been published hitherto (3, 5, 8). Other authors, such as Edelson (4), Dukor (6), Siveira (9) have examined rosette formation on frozen sections which, in our experience, gave consistently negative results, interpretable only on lymphoid tissue (lymph node and spleen).

The technique of extraction gives a variable yield of lymphocytes, ranging from 5×10^5 to 2×10^6 cells/ml. Many factors influence the number of cells extracted, such as the density of these cells or the location of the infiltrate (histologically controlled in every case).

T lymphocytes were detected with the E rosette technique and B lymphocytes with three different markers: EAC RFC, Fab peroxidase, and FITC conjugates. We would stress that filtration through the cotton column does not appear to affect the T/B ratio, as similar results have been obtained with the same tissue specimens both before and after this procedure. So far, the column has proved useful in getting rid of the debris and gives a low contamination rate of macrophages and polynuclear cells. We did not take into account monocytes and polymorphonuclear cells that have both membrane Ig and C3 receptors. These cells can be

distinguished from B lymphocytes in light and electron microscopy (1).

Fab peroxidase conjugate in electron microscopy requires a great number of cells. This technique has only been performed on four specimens. Even so, it appears that B lymphocytes in the tissues (Bti) have two different aspects, one a microvillous membrane bearing surface Ig and the other (Bti) located within the lesions and presenting a smooth membrane and both peripheral and cytoplasmic Ig. This aspect correlates with the count of FITC-Ig bearing lymphocytes.

Bti lymphocytes are always associated with numerous T lymphocytes surrounding BCC, suggesting a B and T cell cooperation which could play a part in an immune defense mechanism. The results obtained in 8 biopsies of delayed hypersensitivity reaction to tuberculin gave us an indication of the relative quantities of B and T cells involved in a classical cutaneous reaction of cellular immunity. Almost total cell labelling was obtained, with a T/B ratio of 5.3. In the BCC infiltrates, the yield of labelled cells fell to 75%, with a T/B ratio of 4.3. 25% of the BCC cell infiltrates failed to take up the marker stains used in this study. Further data are needed to elucidate this fact. A similar T/B ratio, close to that of DHR, has been found in other skin tumours such as squamous cell carcinomas (3).

Cell-mediated immunity is well known as representing the main defense mechanism against tumour proliferation (10). This investigation represented one means of elucidating its role in BCC.

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