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Morphological, immunological and immunocytochemical identification of lymphocytes extracted from cutaneous infiltrates

A. L. CLAUDY, D. SCHMITT, J. VIAC, A. ALARIO, M. J. STAQUET & J. THIVOLET *Laboratoire d'Immunopathologie, Clinique Dermatologique, Hôpital E. Herriot, Lyon, France*

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SUMMARY

The identification of mononuclear cells extracted from various skin disorders has been investigated and the relative amount of B and T cells determined according to various assays such as E-rosette test, EAC-rosette test, FITC and peroxidase anti-immunoglobulin conjugates. It has been found that: (a) there were two different patterns of distribution of the cells in the infiltrates; one with a T/B ratio above 3 (closest to a delayed type reaction), the other with a T/B ratio below 2. (b) The morphological appearance of tissue lymphocytes differs from that of circulating lymphocytes by the presence of both membrane and cytoplasmic immunoglobulins.

INTRODUCTION

Different assays are now available to identify B and T cells in the peripheral blood. Mononuclear cells extracted from the tissues can be submitted to the same assays as circulating cells. To carry out this study, we used various properties of the cell membrane of mononuclear cells, such as for T cells the capacity to form spontaneous rosettes with sheep red blood cells (E–RFC) (Wybran, Carr & Fudenberg, 1972) and for B cells the detection of surface immunoglobulins (Ig) (Froland & Natvig, 1971) and receptors for C3b (EAC–RFC) (Bianco, Patrick & Nussenzweig, 1970). Phagocytic mononuclear cells also bear receptors for C3 but are distinguished by the presence of receptors for IgG1 and a characteristic morphology (Huber *et al.*, 1968).

This paper describes the application of these methods to the study of lymphocytes extracted from normal lymphoid tissue and from cutaneous infiltrates.

MATERIALS AND METHODS

Patients. The mononuclear cells were obtained from 1 cm square skin biopsies taken from a group of twenty-three patients with miscellaneous skin diseases: eight basal cell carcinomas, two squamous cell carcinomas, two nodular melanomas, two lichen planus, one cutaneous leichmaniasis, six delayed hypersensitivity type reactions (DHR), one lymphosarcoma, one acute lymphoblastic leukaemia.

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

Control studies were made on normal circulating lymphocytes and on normal human lymphoid tissue (foetal thymus, lymph nodes, spleen and tonsils).

Isolation of circulating lymphocytes. Forty millilitres of peripheral blood were drawn into a syringe containing 250 u of heparin. The lymphocytes were isolated on a layer of Ficoll Metrizoate by centrifugation at 500 *g* for 30 min. They were washed three times with phosphate-buffered saline (pH 7·2) and the final concentration adjusted according to the different assays:

2×10^6 cells/ml for EAC rosettes and for blood smears using Fab–peroxidase conjugate. 2×10^7 cells/ml for FITC anti-immunoglobulins and E rosettes. All sera were heat-inactivated and absorbed against sheep red blood cells and human erythrocytes.

Extraction of lymphocytes from tissues. Tissue specimens were minced with scissors and scalpel into

Correspondence: Dr A. L. Claudy, Pavillon R, Hôpital E. Herriot, 69374, Lyon Cedex 2, France.

Hanks's balanced salt solution (pH 7·2). The minced tissue and supernatant fractions were filtered through a cotton column. The cells were centrifuged at 500 *g* for 10 min at 4°C. Contaminating red blood cells were lysed with ammonium chloride. After washing cells were adjusted to give a suitable number of lymphocytes for the different tests. Ninety per cent of the cells excluded Trypan Blue.

Rosette formation on circulating and extracted lymphocytes. E rosettes. 0·1 ml of a 2×10^7 cells/ml suspension of lymphocytes was incubated at 37°C for 1 hr with 0·1 ml of foetal calf serum. Sheep red blood cells were added at a concentration of 10^2 per lymphocyte. The mixture was spun at 300 *g* for 3 min and incubated for 12 hr at 4°C. The cells were then re-suspended 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 three red blood cells calculated.

EAC rosettes. One volume of 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 diluted1/5 in veronal-buffered saline was added and incubated at 37° for 30 min. EAC complexes were washed and adjusted to a concentration of $1 \cdot 10^8$ red blood cells per ml. EAC complexes and the suspension of lymphocytes ($2 \cdot 10^6$ cells/ml). Cells were re-suspended on a whirlmixer and EAC rosettes were counted the same way as the E rosettes.

Immunofluorescence (IF). IF staining of lymphocyte surface Ig was made 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 hr at 4°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°C for 3 hr. After an overnight wash in cacodylate buffer, 1 mm^3 blocks were postfixed with 1% osmium tetroxide in sodium cacodylate buffer 0·4 M for 2 hr at 4°C. After rinsing in water and dehydration in alcohol, the tissue fragments were soaked in epoxy resin for 3 days. After polymerization at 60°C for 3 days, the blocks were cut with a Reichert ultramicrotome. One micron semi-thin sections 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 uranylacetate–lead citrate and examined with a Philips EM 300 Hitachi HU 12-A electron microscope.

Histological examination. The samples were fixed directly in Baker fixative and embedded in paraffin. They were then stained with haematoxylin–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, washing with 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 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 glycerine as the mounting medium and examined with a × 100 immersion lens. These preparations could be stored (Avrameas, 1970).

Electron microscopical examination of cell pellets. The general fixation methods used for the preparation of material for routine ultrastructural examination with some alteration in the duration of the different steps was followed. The cells were fixed in suspension with paraformaldehyde for 20 min then washed and put in contact with the conjugate for 3 hr at 37°C. After washing and fixation, the peroxidase activity was demonstrated by reacting with 3,3′-diaminobenzidine for 1 hr in the dark. After washing the cells were postfixed 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

(1) *Blood and normal lymphoid tissues* (*Table* 1)

The cells taken from blood and various lymphoid tissue were subjected to a variety of tests in order to compare the results with those described in the literature (Jondal, Holm & Wigzell, 1972; Yu *et al.*, 1973). The quantitative results given in Table 1 were established according to the percentages of cells forming E and EAC rosettes and bearing various immune markers. The fluorescence of cells labelled by the FITC anti-Ig conjugate could either be in dispersed spots associated with the membrane or forming a continuous circle of fluorescence around the edge of the cell. Cells having a homogeneous cytoplasmic fluores-

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

	E-RFC* (%)	EAC-RFC* (%)	FITC anti-Ig (%)			Fab perox.† (%) light microsc.
			G	M	A	
Peripheral blood	60	12	14	6	2	10
Thymus	95	0	0	0	0	1
Lymph node	30	25		23		n.d.
Spleen	28	34	20	11	3	n.d.
Tonsil	22	50		42		n.d.

n.d. = Not determined.

* RFC: rosette-forming cell.

† Fab perox.: Fab–peroxidase conjugate.

cence and considered to be plasma cells were not taken into account when calculating the percentages.

Cells stained by the immunoperoxidase method, examined in the light microscope, were surrounded by a thin brown continuous band of staining which was comparable to the appearance of the continuous peripheral fluorescence. Discreet patches of stain around the membrane were not seen. Likewise the cells which had a homogenous heavy brown cytoplasmic staining and an eccentric nucleus were not taken into account as they were considered to be plasma cells. A few polymorphonuclear leucocytes and monocytes with a fine cell staining also had to be eliminated. When examined in the electron microscope the immunoperoxidase staining clearly distinguished the two different types of lymphocytes circulating in the blood (Fig. 1). The B cells were rounded with a high nucleo-cytoplasmatic ratio. The nucleus was round and only slightly indented, the peripheral heterochromatin was very dense, the cytoplasm contained few organelles and the membrane was thrown into numerous villi. The stain was dense and continuously distributed on the cytoplasmic

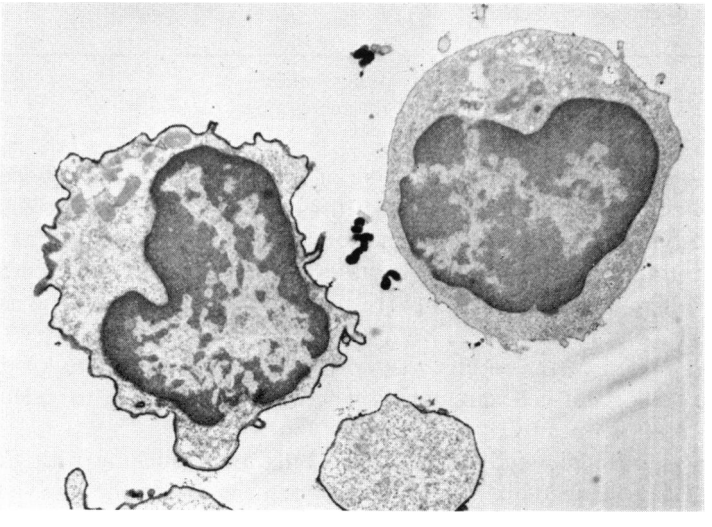

FIG. 1. Circulating lymphocytes (Fab–peroxidase conjugate). Left, B-type lymphocyte with a microvillous membrane, surrounded by a thin continuous band of staining; right, T-type lymphocyte with a smooth membrane. Note absence of peripheral labelling. (Magnification × 6000.)

membrane without labelling the cytoplasm. T lymphocytes had the same general structure of organization of their nuclea cytoplasm but with a smooth surface without any labelling by immunoperoxidase either on the membrane or intracytoplasmic (Reyes *et al.*, 1974) (Fig. 1).

(2) *Cells extracted from cutaneous infiltrates* (*Table* 2)

The cells extracted from the skin were mainly tested by their capacity to form E and EAC rosettes and by their staining by peroxidase conjugate and examination by light microscopy. Very few of the biopsies were able to provide a sufficient number of cells to use all these different methods. The choice for the preferential use of the peroxidase method compared to immunofluorescence will be described later. The quantitative results shown in Table 2 are the preliminary studies obtained in twenty-three patients who had a variety of dermatoses.

TABLE 2. Mean percentage of subpopulations of lymphocytes in various cutaneous infiltrates and blood

	Number of cases	E-RFC (%)		EAC-RFC (%)		Fab-peroxidase conjugate(LM)x (%)	
		Skin	Blood	Skin	Blood	Skin	Blood
Delayed-type skin reactions	6	79	61	15	12	n.d.	
Basal cell carcinoma	8	60	50	14	21	15	9
Squamous cell carcinoma	2	65	51	14	7	n.d.	
Leishmaniasis	1	68	n.d.	18	n.d.	n.d.	
Lichen planus	2	53	63	28	7	n.d.	
Melanoma	2	30	29	45	10	n.d.	6
Lymphosarcoma	1	11	9	3	7	1	2
Acute lymphoblastic leukaemia	1	1	1	21	12	1	1

n.d. = Not determined.
LM(x): light microscopy.

The morphological appearance of labelling of extracted cells with Fab–peroxidase conjugate differs slightly from the appearance of the circulating cells. It was found that some cells, similar to lymphocytes isolated from blood, had a labelling that was limited to the cytoplasmic membrane, but other lymphocytes appeared to have both membrane labelling and a slight labelling of the peripheral parts of their cytoplasm. The same aspect has been found with FITC conjugates.

Statistical evaluation of the results was only possible for specimens of delayed hypersensitivity tests and basal cell carcinomas where it was possible to calculate the 95% confidence limits of the mean values (Table 3).

The electron microscopic study of lymphoid cells after labelling with Fab–peroxidase conjugate could only be made in a few biopsy specimens due to the low yield of cell. The ultrastructural appearance of tissue lymphocytes labelled in this manner was clearly different to that of normal B cells (Figs 2 and 3). They lost their microvilli, there was less localization of Fab at the surface membrane and dense intracytoplasmic labelling occurred comparable to that in plasma cells. These cells retained the main characteristics of lympho-

TABLE 3. Confidence limits of the mean values of E and EAC–RFC in cases of delayed hypersensitivity reactions (DHR) and basal cell carcinomas

	Number of cases	E–RFC (%)		EAC–RFC (%)	
		Mean	Range	Mean	Range
DHR	6	79	74–84	15	11–19
Basal cell carcinoma	8	60	55–65	14	11–17

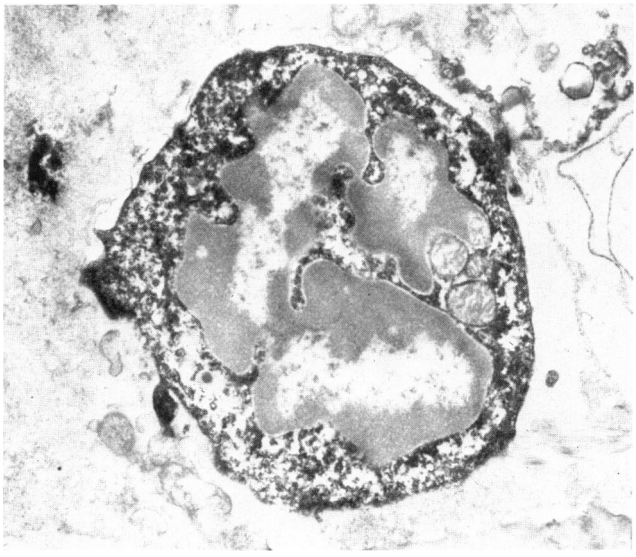

FIG. 2. Extracted lymphocytes (Fab–peroxidase conjugate). Note absence of microvilli and membrane and cytoplasmic Ig. (Magnification ×9000.)

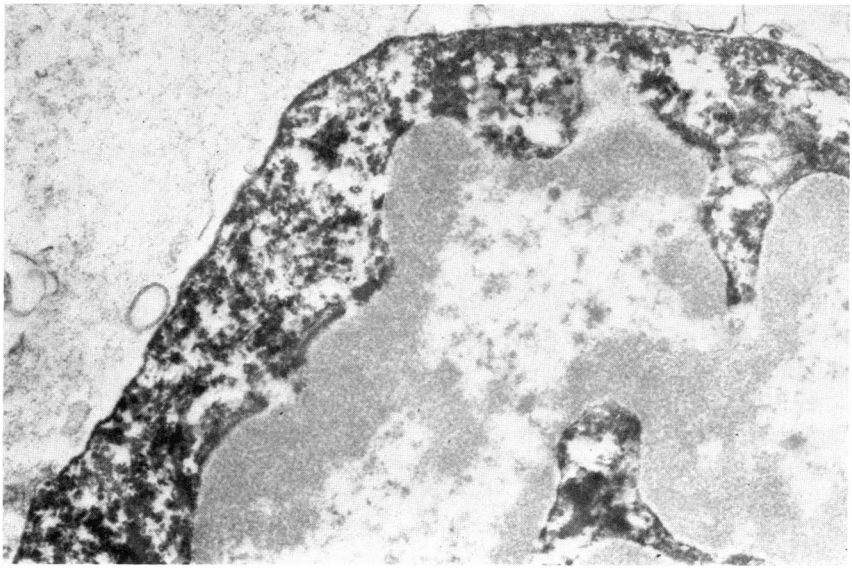

FIG. 3. Detail of Fig. 2. (Magnification ×42,000.)

cytes and were closely allied to B cells by the presence of globulin on their surface membrane. The same aspect was found on tissue section in electron microscopy (Fig. 4). Consequently the extraction procedure does not create an artifact. For the time being, until they can be formally identified we propose to call these cells tissue B lymphocytes (B ti). T lymphocytes that were not labelled and had a smooth membrane were easily recognized in the preparation.

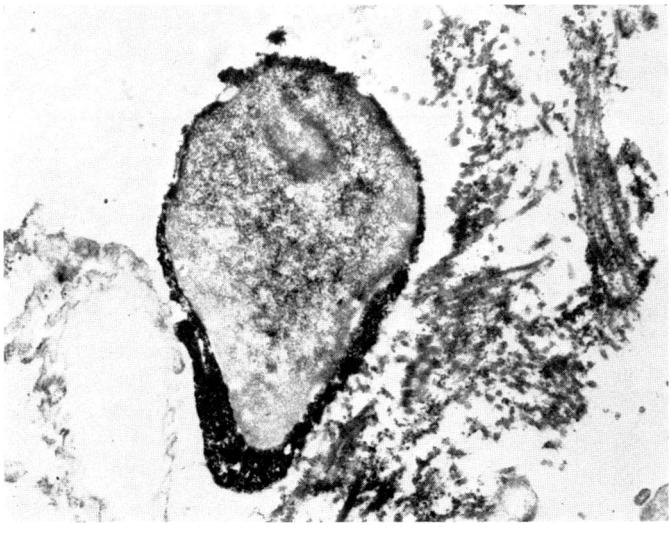

FIG. 4. Tissue lymphocyte in a skin section. Note same appearance as in Fig. 2. (Magnification ×6000.)

DISCUSSION

The technique of extraction of cells described above, gives a variable yield of lymphocytes, sometimes reaching 10^7 cells especially in the cutaneous infiltrates of lymphomas. Thus, in this case all the studies could be carried out. When the yield of cells fell to 2×10^6, only E–RFC, EAC–RFC and Fab–peroxidase staining could be performed. Below this number of cells, E and EAC–RFC alone could be done. In practice, thirty-nine out of fifty trials gave at least 10^5 cells each. Many factors influence the lymphocyte yield such as the density of cells or the superficial location of the infiltrate (histologically controlled in every case). The yield of cells obtained from erythema nodosum nodules was very low, compared to that of basal cell carcinomas. The Fab–peroxidase conjugate technique which can be done with 10^4 lymphocytes was chosen to study the low yield specimens. (IF staining needs 2×10^6 cells/0·1 ml.)

Control studies were performed on peripheral lymphocytes, thymus, lymph nodes, spleen and tonsils (Table 1); the results corroborate those of the literature (Yu *et al.*, 1973; Edelson *et al.*, 1973). A small percentage of cells, especially in lymphoid tissues and blood, seem to lack specific markers. This has been stressed previously (Ross, Rabellino & Polley, 1973). The reasons for the absence of staining of certain lymphocytes in the different markers described in this paper will not be discussed. The identification of T lymphocytes has been performed with only one marker (E–RFC).

B lymphocytes were detected with three different markers: EAC–RFC, IF, and Fab peroxidase conjugate. Fab peroxidase conjugate gives a percentage of staining constantly lower than that of IF or EAC–RFC. The technique is less accurate than IF.

This lack of sensitivity strongly suggests a low diffusion rate of Fab–peroxidase molecules.

On the other hand, the method has two advantages, the stability of the staining which allows the smears to be kept indefinitely and a very few cells are required to make the test. In some cases, when the cells are sparse, it may represent the only technique available to stain membrane Ig. EAC–RFC and membrane-Ig-bearing cells are not only B lymphocytes. Monocytes and polymorphonuclear cells also have membrane Ig and C3 receptors, but these cells can be distinguished in light and electron microscopy (Abramson *et al.*, 1970). A close observation of the stained cells can avoid errors. Furthermore, the technique of cell separation excludes substantial contamination by macrophages or polynuclear cells.

Fab–peroxidase conjugate in electron microscopy requires a great number of cells and could only be performed on a few specimens that have a high density of cells or when this was the sole examination. Despite the few specimens studied, some features can be stressed. There was a clear-cut correlation between the percentage of E–RFC and T lymphocytes. AB lymphocytes in the tissues (B ti lymphocytes) have a distinct appearance. This aspect seems to correspond to most of the membrane Ig bearing lymphocytes and EAC–RFC. A study is now in progress to try to identify EAC–RFC with ultrastructural immunocytochemical staining. B lymphocytes may have two different appearances; one with a microvillous membrane, bearing surface Ig and circulating; the other (B ti) located within the lesions and presenting a smooth membrane and cytoplasmic Ig. This concept is in agreement with the results of Fab–peroxidase conjugate staining in light microscopy. The finding that the B ti lymphocyte has a smooth membrane further supports the idea that the structural modifications are secondary to cell migration through a dense population of cells and collagen, and are not due to the fixation procedure since the cells are prefixed before processing. B ti lymphocytes are associated with a high percentage of T lymphocytes in the lesions, suggesting that B and T lymphocytes are able to co-operate within the tissues.

Until now, only a few studies of this type have been published (Schmitt *et al.*, 1975). None of the previous studies demonstrated the distribution of immunoglobulins by electron microscopy. Edelson *et al.* (1973) and Dukor, Bianco & Nussenzweig (1970) examined EAC rosette formation on frozen sections which in our experience only give interpretable results in lymphoid tissues (nodes and spleen). This technique was consistently negative on frozen sections of skin, as well as the E rosette formation (Silveira, Mendes & Tolnai, 1972).

Study of various pathological lesions

This is a preliminary communication, mainly to illustrate the technique, rather than the particular results obtained in the individual biopsies. More detailed studies are at present being completed, in the meantime there are some points that can be discussed. The results

TABLE 4. E/EAC ratio (mean value) of infiltrative cells in various skin disorders compared to delayed hypersensitivity reactions

		E–RFC (%)	EAC–RFC (%)	E/EAC ratio
	Delayed-type skin reactions	79	15	5·3
E/EAC < 3	Basal cell carcinoma	60	14	4·3
	Squamous cell carcinoma	65	14	4·6
	Leishmaniasis	68	18	3·8
	Lymphosarcoma	11	3	3·7
E/EAC < 2	Melanoma	30	45	0·7
	Lichen planus	53	28	1·8
	Acute lymphoblastic leukaemia	1	21	0·0

obtained in six biopsies of the DHR to tuberculin gave an indication of the relative amounts of B and T cells in the classical cutaneous reactions of cellular immunity. Labelling of nearly 100% of the cells was obtained with an increased percentage of T cells ($79 \pm 5\%$) and a T:B ratio of 5·21:1. This will be studied further by comparing the T- and B-cell populations in various skin reactions to irritation. The results can be divided according to the extent to which they approach or diverge from the model of DHR. There are two groups (Table 4): a population which is closest to a DHR:% T cells (E rosettes) 60% and an E/EAC ratio >3. Within this group there were the basal cell carcinomas (4·3) a squamous cell carcinoma (4·6) and a case of leishmaniasis (3·8). The second population differs from that in DHR with a percentage of T cells (E rosettes) of 30–35% and an E/EAC ratio <2. In this group were lichen planus (1·8) and malignant melanomas (0·6). It is unwise to suggest any interpretation; possibly, it indicates a DHR in benign basal cell tumours and its absence in extensive malignant melanomas.

As very few patients with cutaneous localizations of haematological malignancies were studied, little can be said about them at this stage, further work is needed as they raise different problems to those in the main part of our study.

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