

IN SITU IDENTIFICATION OF MONONUCLEAR CELLS IN CUTANEOUS INFILTRATES IN DISCOID LUPUS ERYTHEMATOSUS, SARCOIDOSIS AND SECONDARY SYPHILIS

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Abstract. The inflammatory mononuclear cell infiltrates observed in lesional skin from patients with discoid lupus erythematosus (DLE), sarcoidosis, and secondary syphilis have been characterized *in situ*. Immunological markers (human T lymphocyte antigens and receptors for sheep erythrocytes, C3b, C3d and Fc γ) were studied by using immunofluorescence tests with IgG F(ab')₂ preparation of anti-T lymphocyte serum, hemadsorption with tissue sections, and tests with soluble immune complexes of peroxidase.

In DLE, T lymphocytes were the dominant cell type. In sarcoidosis, the epithelioid cells, including giant cells, had markers similar to macrophages. The lymphohistiocytic cells consisted mainly of macrophages, some T lymphocytes and a few B lymphocytes. In secondary syphilis (condylomata lata), macrophages and T lymphocytes were the dominant cell types, and relatively few B lymphocytes were detected.

Key words: Discoid lupus erythematosus; Sarcoidosis; Secondary syphilis; Skin sections; Mononuclear cells; Immunological markers

In recent years progress has been made in developing immunological techniques for the *in situ* study of inflammatory mononuclear cells observed in skin diseases. T lymphocytes can be demonstrated by their capacity to bind sheep erythrocytes (E) treated with 2-aminoethylisothiuronium bromide hydrobromide (AET), (E_{AET}), using a closed chamber technique (4, 5), and by specific human T lymphocyte antigens (HTLA) (7, 24). B lymphocytes possess receptors for the complement (C) components C3b and C3d (2, 20), while macrophages have receptors for C3b only (20, 25). These C3 receptors can be demonstrated by using E sensitized with rabbit antibody (A) of IgM class and coated with either C3b (IgMEAC3b) or C3d (IgMEAC3d) (4, 5, 16). B lymphocytes and macrophages also have receptors for the Fc portion of the IgG molecule (FcR) (1, 10). FcR can be dem-

onstrated either by E sensitized with various amounts of rabbit IgG antibody (IgGEA) (4), or by soluble complexes of horseradish peroxidase (HRP) and rabbit IgG antibody to HRP (15) (Table 1).

We have studied the mononuclear cell infiltrates in cryostat sections of lesional skin from patients with etiologically and clinically differing dermatoses. The results obtained with the different methods were compared and evaluated.

MATERIALS AND METHODS

Patients

Six patients with chronic discoid lupus erythematosus (DLE), 3 patients with chronic cutaneous sarcoidosis and 1 patient with secondary syphilis (condylomata lata) were studied. Routine histology confirmed the clinical diagnoses of DLE and sarcoidosis. In addition, there were typical direct immunofluorescence findings, with granular deposits of IgG and C3 in the basal membrane zone, in sections of lesions from the patients with DLE. The tuberculin skin test was negative in all patients with sarcoidosis. Two of the patients showed involvement of sarcoidosis also in lungs and lymph nodes. *Treponema pallidum* was identified in serous exudates from condylomata lata by dark-field examination in the patient with secondary syphilis.

Tissues

Elliptical skin biopsies, approximately 5×15 mm in diameter, were taken from lesions and uninvolved skin of all patients. The specimens were quick-frozen and sectioned (4-6 μ m) as described elsewhere (5). Sections from all frozen specimens were stained with hematoxylin and eosin. Serial cryostat sections were examined for comparison of the density of the cell infiltrates and positivity of the reactions.

Specimens of normal spleen, kidney, and thymus were obtained from the Department of Surgery.

Erythrocytes

Erythrocytes were handled as described previously (5).

Table 1. Identification of mononuclear cells in tissue sections by using various immunological markers

Cell	Marker	Indicator system
T lymphocyte	E receptor HTLA ^b	E _{AET} ^a Anti-T lymphocyte serum
B lymphocyte, macrophage	C3b receptor	IgMEAC3b ^c
B lymphocyte	C3d receptor	IgMEAC3d ^d
B lymphocyte, macrophage	Fcγ receptor	IgGEA ^e Soluble immune complexes

^a E_{AET} = sheep erythrocytes treated with AET.

^b HTLA = human T lymphocyte antigen.

^c IgMEAC3b = sheep erythrocytes sensitized with IgM antibody and C3b.

^d IgMEAC3d = IgMEAC3b treated with C3d inactivator.

^e IgGEA = sheep erythrocytes sensitized with varying amounts of IgG antibodies, expressed in agglutinating units.

Sera

Fresh normal human serum from blood donors was used as the source of C. Inactivated serum (56°C for 30 min) absorbed with sheep erythrocytes (E) was used as C3b inactivator reagent (22).

Antiserum to E was raised by immunizing rabbits. The rabbits were bled at various intervals after immunization to obtain samples containing IgM and IgG antibodies.

Anti-human T lymphocyte serum was raised by immunizing rabbits with human thymocytes (17).

Rabbit antiserum to HRP (type IV, Sigma, St. Louis, Mo., USA) was raised as described previously (15).

Immunoglobulin preparations

Preparations of rabbit IgG and IgM antibodies to E were obtained by gel filtration on Sephadex G200 equilibrated with phosphate-buffered saline, pH 7.2 (PBS). The agglutinating activity of the IgG and IgM fractions against E was examined before and after treatment with mercaptoethanol to destroy the IgM.

Fluorescein isothiocyanate (FITC)-conjugated porcine antibodies to rabbit immunoglobulins were purchased from DAKO-immunoglobulins a/s, Denmark (code no. F 2 190, molar F/P ratio 2.3) as also were FITC-conjugated rabbit antibodies to human IgG (code no. F 1 090, molar F/P ratio 2.3).

IgG antibody to HRP was purified from the rabbit anti-HRP serum on HRP-coupled Sepharose 4B (Pharmacia, Uppsala, Sweden) activated with cyanogen bromide as described elsewhere (15). Rabbit F(ab')₂ was prepared as described by Stewart et al. (26).

IgG F(ab')₂ preparation of the anti-human T lymphocyte serum was obtained and rendered specific as described recently (17).

The protein concentrations of the preparations were calculated assuming $E \frac{1\%}{1\text{cm}} = 280 \text{ nm} = 14.6$. The preparations were filtered through a sterile 0.45 μm Millipore filter before use.

Immune complexes

Immune complexes were prepared by adding dilutions of the antibody or the preparation of F(ab')₂ fragments to HRP to equal amounts of four-fold dilutions of HRP from 1 mg/ml in PBS. The mixtures were incubated for 2 hours at room temperature before use. In tests with tissue sec-

tions, complexes prepared at slight antigen excess were used (15).

Indicator cells

E_{AET} was prepared according to the procedure described by Kaplan & Clark (14). The E_{AET} was used as a 1% suspension in PBS containing 25% fetal bovine serum, stored at 4°C and used within 5 days.

IgMEAC3b and IgMEAC3d indicator cells were prepared as described elsewhere (5). Briefly, E were sensitized with $\frac{1}{2}$ agglutinating unit of rabbit IgG antibody (IgMEA), washed and made up to a 1% suspension in barbital (Veronal)-buffered saline, pH 7.2, containing 0.15 mM CaCl₂, 0.5 mM MgCl₂ and 0.01% gelatin (GVB). One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of a 1% suspension of E. Equal volumes of 2% IgMEA and zymosan-treated C diluted 1:8 in GVB were mixed, incubated for 10 min at 37°C, washed and made up to a 1% suspension in GVB (IgMEAC3b). One ml of a 1% suspension of IgMEAC3b in GVB was mixed with 2 ml of C3b inactivator reagent, washed, incubated at 37°C for 2-4 hours, washed and finally made up to a 1% suspension in GVB (IgMEAC3d).

IgGEA were prepared as described previously (4), and used as a 1% suspension in PBS containing 2% NaCl. E was also sensitized similarly with preparation of F(ab')₂ fragments of IgG anti-E.

Hemadsorption with tissue sections

The indicator cells were applied to the tissue sections by using a closed chamber technique as described by Tønder et al. (29). In tests with E_{AET} the sections were incubated with the indicator cells overnight at 4°C before the slides were turned over and read microscopically while still cold. In other tests the sections were incubated with the indicator cells at room temperature for 20 min. In strongly positive reactions (3+) the reacting tissue was covered by erythrocytes in a tight monolayer, and in moderately positive reactions (2+), partially covered. In weakly positive reactions (1+), only scattered adhering indicator cells were observed. When no erythrocytes attached to the tissue, the reaction was termed negative (-).

The preparations were fixed and stained as described previously (5).

Table II. Results obtained with different indicator cells, anti-T lymphocyte serum and complexes of peroxidase, tested with sections of lesional skin from patients with discoid LE, sarcoidosis and secondary syphilis

Percentage of cells stained: >70% = 3+, 30-70% = 2+, <30% = 1+. No reaction = -

Patients	Indicator systems				IgGEA		Complexes of peroxidase
	E _{AET}	Anti-T serum	IgMEAC3b	IgMEAC3d	1 agg.	½ agg.	
Discoid LE							
J. L. ^a	3+ ^b	3+ ^c	2+	1+	3+	1+	1+ ^c
I. S.	3+	3+	1+	-	2+	1+	1+
N. H.	3+	3+	2+	1+	2+	1+	1+
T. O.	2+	3+	2+	-	2+	1+	1+
S. B.	2+	3+	1+	-	1+	-	1+
B. A.	2+	3+	1+	-	1+	-	1+
Sarcoidosis							
J. K.	1+	2+	3+	1+	3+	2+	3+
P. N.	1+	1+	3+	1+	3+	2+	3+
J. L.	2+	2+	3+	1+	3+	2+	3+
Secondary syphilis							
P. G. F.	2+	3+	3+	1+	2+	1+	1+

^a The initials of the patients.

^b See Materials and Methods for explanation of grading of reaction with indicator cells.

^c Grading of reaction with anti-T lymphocyte serum and immune complexes of peroxidase.

Binding of HRP-anti-HRP immune complexes

Cryostat sections were incubated with the immune complexes in a moist chamber at room temperature for 30 min and washed in PBS. The peroxidase activity was revealed by incubating the sections in Graham & Karnovsky's solution (13). Some sections were then incubated with 1% OsO₄ for 30 sec at room temperature. All of the sections were washed in PBS, stained with hematoxylin and mounted in Depex mounting medium before microscopy reading.

Demonstration of T lymphocytes with anti-T lymphocyte serum

Cryostat sections were washed for 30 min in PBS, and then incubated with the IgG F(ab')₂ preparation (10 mg/ml) diluted 1:4 in PBS for 30 min in a moist chamber at room temperature. The sections were washed for 30 min in PBS in beakers with gentle agitation, incubated for another 30 min at room temperature with FITC-conjugated porcine anti-rabbit Ig diluted 1:20 in PBS and washed for 30 min. The sections were mounted in PBS-glycerol and examined in a Leitz Orthoplan microscope with an Osram HBO-200 mercury lamp.

Demonstration of IgG bound in vivo

Cryostat sections were washed at 4°C in PBS for 2 hours before incubation with FITC-conjugated rabbit antiserum to human IgG diluted 1:32. The sections were then washed for 30 min, mounted and examined as described above.

Controls

The reactivity of E_{AET} and the IgGEA was tested with tissue sections of specimens from normal human spleen (5, 12). The IgMEAC3b and IgMEAC3d were tested with tissue sections of specimens from both spleen and kidney. The IgMEAC3b adhere within both the white and red pulp of spleen, while the IgMEAC3d adhere to the white pulp areas only (12). Glomeruli in human renal tissue have receptors for C3b only (16). Immune adherence tests with human O Rh-negative erythrocytes served as an additional control of reactivity of IgMEAC indicator cells. IgMEAC3b induce immune adherence of primate erythrocytes, while IgMEAC3d do not (20, 23). E sensitized with F(ab')₂ fragments. IgMEA and IgMEA incubated with zymosan-treated human serum did not adhere to any of the sections.

Complexes of rabbit IgG anti-HRP and HRP were bound strongly to the same areas of human spleen sections as were IgGEA. Complexes containing F(ab')₂ fragments of IgG anti-HRP or HRP solution did not stain the cell membranes in any of the infiltrates. However, some cells were stained when sections were incubated with Graham & Karnovsky's solution alone. They may represent macrophages, indicating strong endogenous peroxidase activity. These cells could be easily identified as the staining was very dark and localized to the whole cytoplasm.

The IgG F(ab')₂ preparation of rabbit anti-human T lymphocyte serum stained thymocytes in sections of thymus and lymphocytes in the periarteriolar areas in sections of spleen (17). Sections incubated with PBS and FITC-labelled porcine anti-rabbit Ig serum were not stained.

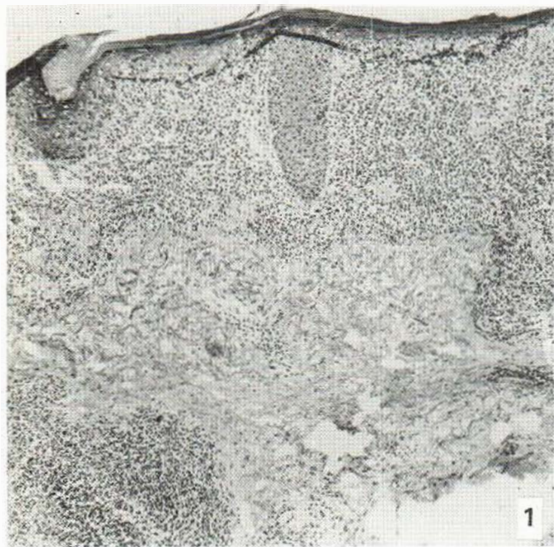


Fig. 1. Skin section of DLE lesion (patient J. L.) showing a dense dermal mononuclear cell infiltrate. H & E. $\times 60$.

Fig. 2. DLE. Section of same lesion as in Fig. 1. showing massive adherence of E_{AFT} mostly as single cells, but many rosettes (arrows) are also seen (unfixed and unstained preparation, $\times 100$).

Fig. 3. Same preparation as in Fig. 2 after fixation and staining with diaminobenzidine and H & E. $\times 100$.

RESULTS

The results obtained in tests with sections of lesional skin from all patients examined are presented in Table II. The quantity of indicator cells that attached to the tissue sections varied according to the density of the dermal cell infiltrates in each dermatoses. No reaction was observed in corresponding areas of dermis in uninvolved skin.

Demonstration of E receptors and HTLA

The strongest reactions with E_{AFT} were obtained with lesional skin from patients with DLE. The indicator cells almost completely covered the infiltrates, partly forming rosette-like figures (Figs. 1-3). Similarly, the strongest reactions obtained in tests with IgG $F(ab')_2$ preparations of anti-T lymphocyte serum were recorded in sections of DLE skin lesions (Table II). The staining with the preparation of anti-T lymphocyte serum was sharply delineated and localized to most of the cell membranes in the infiltrates (Fig. 4). Accordingly, the results obtained

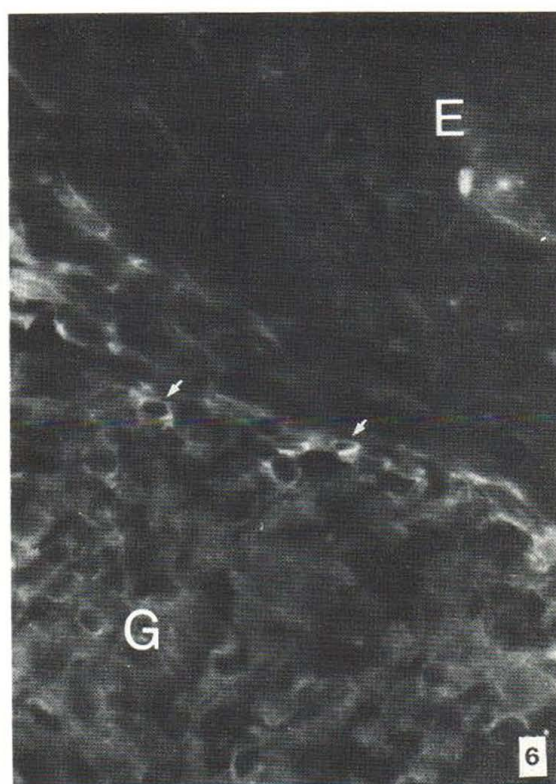
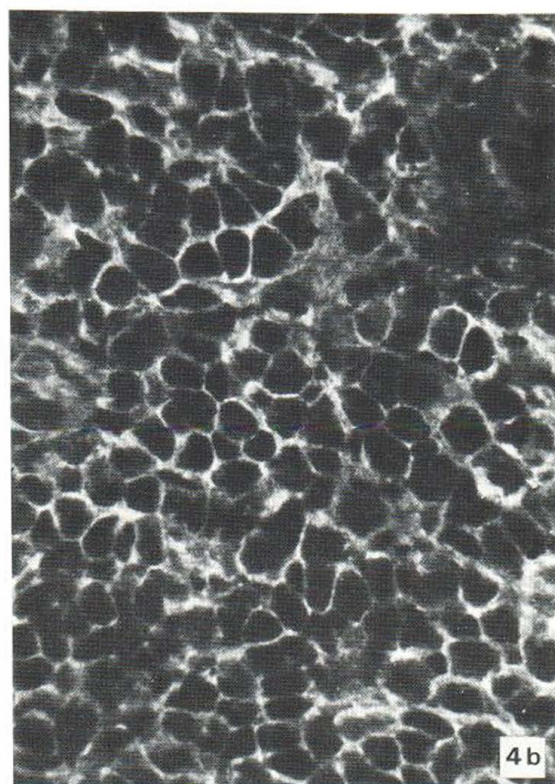
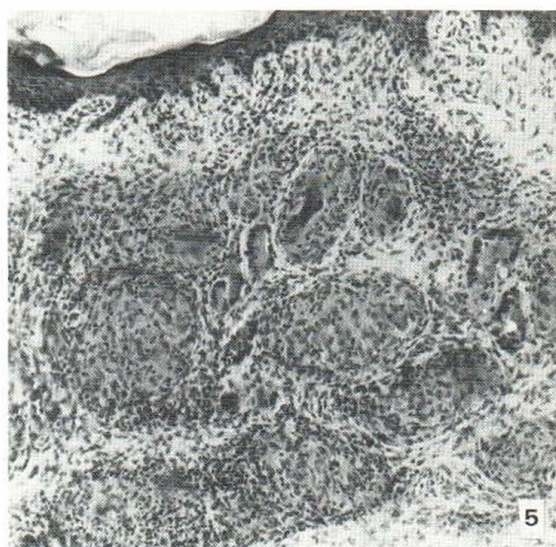
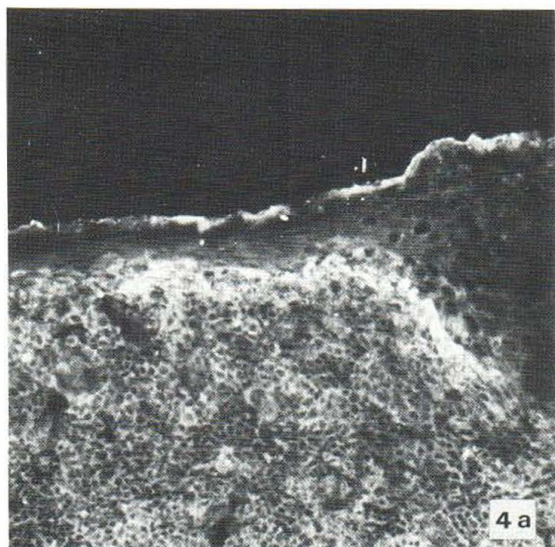


Fig. 4 a-b. Skin section of same DLE lesion as in Figs. 1-3, showing result of immunofluorescence test with IgG F(ab')₂ preparation of anti-T lymphocyte serum. Most of the inflammatory cells are labelled (Fig. 4a, $\times 100$; Fig. 4b, $\times 400$).

Fig. 5. Skin section of sarcoid lesion (patient J. K.) with large islands of epithelioid and giant cells surrounded by a

moderate admixture of lymphohistiocytic cells. H & E, $\times 100$.

Fig. 6. Skin section of sarcoid lesion (patient P. N.) showing staining with anti-T lymphocyte serum. Stained T lymphocytes (arrows) are few and are located mainly in the periphery of a granuloma (G). Epidermis (E) is seen in the upper right part of the figure. $\times 355$.



Fig. 7. Sarcoidosis. Section of same lesion as in Fig. 5, showing massive adherence of IgMEAC3b corresponding to the granulomas. Unfixed and unstained preparation, $\times 100$.

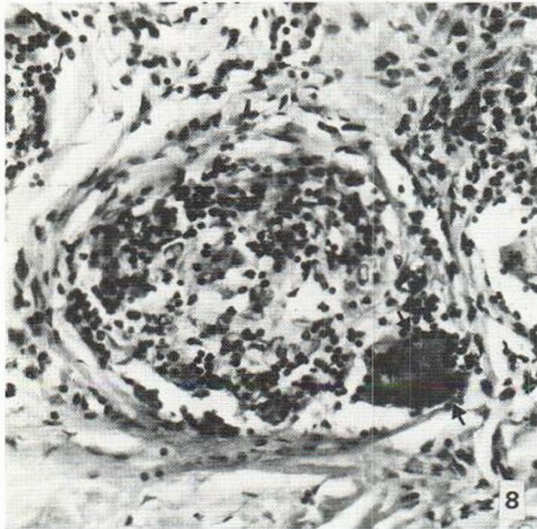


Fig. 8. Section of sarcoid skin granuloma (patient P. N.) showing adherence of IgMEAC3b. Indicator cells adhere along cell membrane of a giant cell (arrows). Diaminobenzidine and H & E, $\times 250$.

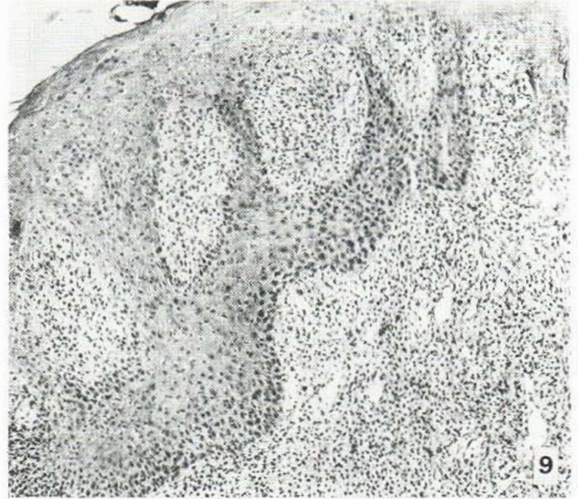


Fig. 9. Section of condyloma latum (patient P. G. F.). Dense infiltration of mononuclear cells in the dermis. H & E, $\times 100$.

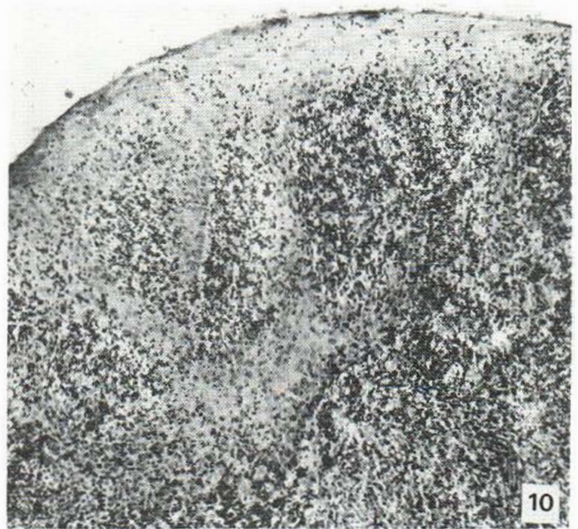


Fig. 10. Condyloma latum. Section of same lesion as in Fig. 9, showing adherence of IgMEAC3b. Diaminobenzidine and H & E, $\times 100$.

with both tests indicate that most dermal inflammatory cells in DLE are T lymphocytes.

Weaker reactions were obtained in experiments with lesional skin from the patient with secondary syphilis. Both hemadsorption with E_{AET} and the staining with preparation of anti-T lymphocyte serum were recorded as moderately positive, indi-

cating the presence of T lymphocytes, though not so numerous as found in DLE.

The weakest reactions of E_{AET} were obtained in tests with sarcoid skin lesions (Figs. 5, 6), where the indicator cells adhered mainly between and in the periphery of the granulomas. Corresponding results were obtained by using the preparation of anti-T

lymphocyte serum. Most of cells in the granulomas were unstained. There was no staining of the giant cells.

Demonstration of C3 receptors

The strongest adherence of IgMEAC3b was obtained with sections from sarcoid skin lesions. The indicator cells adhered to lymphohistiocytic and epithelioid cells in the granulomas and along the cell membranes of giant cells (Figs. 7, 8). Few IgMEAC3d indicator cells adhered to the lesions. The findings suggest that the epithelioid cells are derived from macrophages.

The histology showed that the dermal cell infiltrates in lesions of secondary syphilis consisted mainly of mononuclear cells including many plasma cells. IgMEAC3b indicator cells covered the whole infiltrates (3+ reaction) (Figs. 9,10). Few IgMEAC3d indicator cells adhered to the sections (1+ reaction), indicating that most of the inflammatory cells are macrophages. In lesions of DLE the IgMEAC3b test gave moderately positive reactions in sections from 2 patients and weakly positive in the other 3. Only a few IgMEAC3d indicator cells adhered. The results are consistent with the presence of some macrophages and very few B lymphocytes.

Demonstration of FcR

The results of the IgGEA test are dependent on the amount of A used for the sensitization of E, i.e. stronger sensitization results in stronger reactions. E sensitized with 1 and with $\frac{1}{2}$ agglutinating unit was used, as these indicator cells gave 3+ and 2+ reactions respectively with sections of spleen.

Strongest adsorption of IgGEA was recorded in tests with sections of lesional skin from patients with sarcoidosis (Table II). The strongest reactions were located in the granulomas. Similar results were obtained by using soluble complexes of peroxidase. Nearly all the epithelioid cells, including giant cells, were stained (Fig. 11). The findings substantiate the concept that these cells have receptors similar to macrophages.

In sections of lesions of both DLE and secondary syphilis relatively few mononuclear cells were stained with complexes of peroxidase. In sections of the same lesions, IgGEA adhered sparsely, with only 1+ reaction using $\frac{1}{2}$ agglutinating unit of A. As regards DLE lesions, these findings are in agreement with the results of the C3 receptor tests, indi-

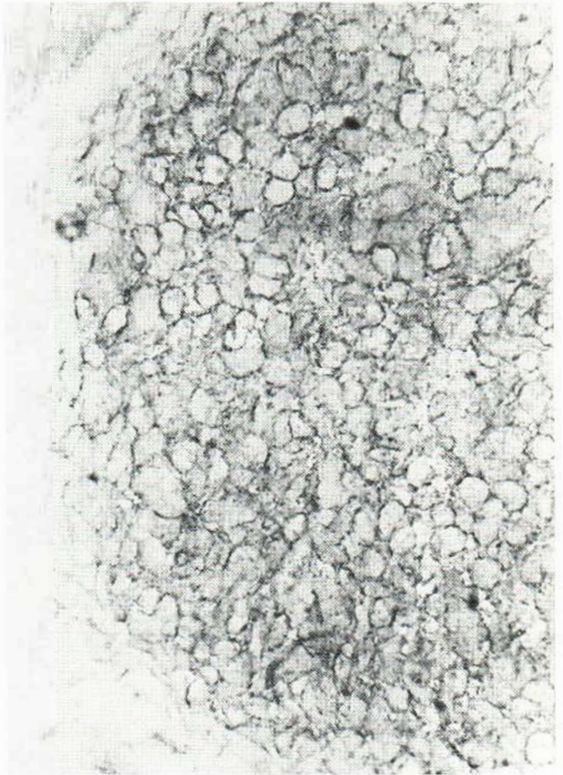


Fig. 11. Sarcoid skin granuloma. Section of same lesion as in Fig. 6. Result of incubation with complexes of HRP-anti-HRP. Bound HRP revealed with diaminobenzidine and OsO_4 is seen along the cell membranes of most cells. Unstained. $\times 355$.

cating a relatively low proportion of macrophages and very few B lymphocytes. As the secondary syphilitic lesions contained numerous macrophages (results of C3 receptor tests), the low FcR activity revealed by tests with IgGEA and complexes of peroxidase suggests partial *in vivo* blocking of FcR.

Demonstration of IgG bound in vivo

After incubation with anti-human IgG, many of the cells in the infiltrates of secondary syphilis revealed a rim-like fluorescence. This may indicate both IgG bound to FcR and IgG as surface marker on B lymphocytes (2). Cytoplasmic fluorescence in some cells indicating IgG-positive plasma cells was also seen.

DISCUSSION

The results showed that tests using E_{AET} hemadsorption and IgG F(ab) $_2$ preparation of anti-T lym-

phocyte serum to demonstrate T lymphocytes *in situ* gave reproducible and comparable results. We found that E_{AFT} using a closed chamber was the most convenient and simple method. Although both methods have to be considered as semi-quantitative the use of a preparation of anti-T lymphocyte serum gave the most pinpointing reactions and this was best suited for quantifying T lymphocytes. However, the use of anti-T lymphocyte serum is more complicated and time-consuming. The specificity of the antiserum prepared has to be carefully ascertained. The use of IgG F(ab')₂ preparation of anti-T lymphocyte serum excludes possible binding to FcR present in the tissue.

Comparable results were obtained with IgGEA hemadsorption and with immune complexes of HRP-anti-HRP to demonstrate FcR-positive cells in tissue sections. In order to quantify FcR-positive cells *in situ*, the use of complexes of HRP-anti-HRP was best. This method which makes possible a precise localization of FcR in tissue, has not previously been applied to skin sections. It is simple, sensitive, reproducible, easy to perform and the stained sections can be preserved. The granular brown reaction product is detectable by conventional microscopy. We used osmification to enhance the contrast for microphotography, but otherwise omitted this procedure because it tends to destroy morphological details of the tissue in cryostat sections. For satisfactory staining it was important to use thin sections (4–6 μm) and only slight hematoxylin staining.

On comparing the results of the different tests, we concluded that 70–80% of the mononuclear cells in the DLE skin lesions examined were T lymphocytes. The results are in agreement with recent findings of others using anti-T lymphocyte serum (8, 24). The proportion of B lymphocytes determined by results of the IgMEAC3d test does not seem to exceed 5–10% in any of the DLE lesions studied.

In sections of sarcoid skin lesions, the epithelioid cells of the granulomas including giant cells had markers similar to macrophages. This is in line with results previously obtained using histochemical staining (18) or, more recently, using indicator cells (IgGEA, IgMEAC) and tissue sections with an 'open' hemadsorption technique (27). Ridley et al. (21) reported that epithelioid cells in sarcoidosis lose the FcR, and Turk (28) advocated that this is characteristic for epithelioid cells. Lack of FcR on

B lymphocytes has also been reported (9, 11). Compared with our results this discrepancy may be explained by differences in the sensitivity of the techniques used. When using the closed chamber technique, unbound cells detach from the tissue sections by gravity alone. Tønder et al. (30) showed that this technique gave considerably stronger reactions with lymphoid organs and tumour tissue than tests with an 'open' technique where indicator cells are washed off in PBS. In the present study the cells in the granulomas were so close together that it was difficult by light microscopy to determine the exact localization of the granular reaction product obtained in tests with complexes of HRP-anti-HRP. Intercellular location of FcR has been demonstrated in mononuclear cell infiltrates in malignant tissue (31). To further elucidate this possibility in sarcoid skin lesions, electronmicroscopy studies are in progress.

The present results indicate that cells of macrophage origin comprise the bulk of the cells in the sarcoid granulomas. There are fewer T lymphocytes and very few B lymphocytes. Most T lymphocytes were detected corresponding to the lymphohistiocytic cells surrounding the epithelioid island. This tallies with a recent report of Chu et al. (8) who used anti-T lymphocyte serum for the *in situ* demonstration of T lymphocytes. On the other hand, Schmitt et al. (24), using the same method, demonstrated a predominant T lymphocyte population in the cutaneous lesions of sarcoidosis, in line with the results obtained by Alario et al. (3) characterizing mononuclear cells liberated from the granulomas. However, the latter authors suggested that the high percentage of T lymphocytes (84%) could be due to loss of adherent cells by the liberating process.

In skin sections of secondary syphilitic lesions (condylomata lata), macrophages and T lymphocytes were the dominant mononuclear cells. At least 50% of the cells appear to be macrophages and 40% T lymphocytes. The proportion of B lymphocytes is estimated to 5–10% as result of the IgMEAC3d test. The number of cells showing membrane-associated IgG was significantly higher than the number of cells bearing receptor for C3d. The low FcR activity, together with this demonstration of IgG, indicated blocking of FcR by *in vivo* bound IgG. Unfortunately, shortage of tissue hampered further studies by elution of IgG from the sections to obtain stronger FcR reactions in order to substantiate this

assumption. To our knowledge, characterization of mononuclear cells *in situ* in skin or mucosal lesions of syphilis has not been performed previously. Previously studies have indicated that cellular immunity is of little importance or may even be depressed in early syphilitic infection (6, 19). The dominance of macrophages and T lymphocytes in the infiltrates, however, may suggest that cell-mediated immune response is of importance in the host-parasite interaction at this stage of the infectious process.

The heterogeneity of mononuclear cells as judged by surface markers, makes it important to use different methods when characterizing the cells. We have used various markers and different methods, thereby increasing the reliability of the *in situ* characterization and quantitation of mononuclear cells. Two different methods were specific for the detection of T lymphocytes and two for FcR. The only test specific for B lymphocytes was the IgMEAC3d test. We have examined several dermatoses by using IgMEAC3d and constantly find a low proportion of B lymphocytes in dermal inflammatory infiltrates (4, 5). We suggest that T lymphocytes are the dominant lymphocytes in all inflammatory infiltrates in the skin, while B lymphocytes are probably present in low numbers only.

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