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An immunohistological analysis of lymphocyte subpopulations and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies

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SUMMARY

We have used monoclonal antibodies of the orthoclone (OKT) series to identify T cell subsets in an immunohistological analysis of the synovial membranes obtained from normal individuals and patients with osteoarthritis or rheumatoid arthritis. T cells of the inducer and the suppressor/cytotoxic subsets were identified by the OKT4 and OKT8 antibodies respectively while HLA-DR (Ia-like) antigens were recognized by a conventional antiserum. In the normal and osteoarthritic synovial membranes, virtually no lymphocytes were identified whereas the mononuclear cell infiltrates of the rheumatoid synovial membranes were composed predominantly of T cells expressing the OKT4 inducer phenotype with few $OKT8^+$ suppressor/cytotoxic cells. The $OKT4^+$ cells were found to be intimately related to B cells and strongly $HLA\text{-}DR^+$ cells which morphologically resembled the interdigitating cells of lymph nodes. The micro-anatomical arrangement of these different cell types in the mononuclear infiltrates of the rheumatoid synovial membranes closely resembled that of the paracortical or T cell dependent area of normal lymph nodes except few $OKT8^+$ lymphocytes were present. These findings are explained in terms of rheumatoid arthritis as a disease of altered T lymphocyte/macrophage immunoregulation.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, the main feature of which is the development of a destructive arthropathy that may involve all synovial joints in the body. Its effects however are not confined to the joints, and other organ systems may be involved. RA has many clinical, laboratory and pathological features in common with other immunologically-mediated and infectious diseases. Of these, the best known are the characteristic mononuclear cell infiltrates seen in the synovial membrane (SM), and the production of autoantibodies such as rheumatoid factor. These observations together with the recently described association of RA with the HLA-D4/DR4 antigen in the Caucasian population (Stastny, 1976; Panayi, Wooley & Batchelor, 1978) have led to the concept of RA as an immunologically-mediated disease occurring in a genetically predisposed individual on exposure to an unknown antigen.

The cellular infiltrate in the SM contains lymphocytes which may be scattered diffusely in the

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SM, arranged in perivascular collections, or follicles; in rare instances true germinal centres are formed (Gardner, 1972). In addition to lymphocytes, plasma cells and macrophages are evident.

Numerous attempts have been made to analyse the lymphocyte subpopulations present in rheumatoid SM using immunofluorescence and cytoadherence techniques. Approximately 80% of the lymphocyte population is composed of T cells, less than 20% are B cells (Froland & Abrahamsen, 1979). The B cells are distributed in the centres of lymphoid follicles and in structures resembling germinal centres (Tannenbaum *et al.*, 1975; Meijer *et al.*, 1977). Within the lymphocytic infiltrates, plasma cells are also seen. In addition, functional studies have shown that rheumatoid SM is a site of immunoglobulin synthesis (Smiley, Sachs & Ziff, 1968) and lymphokine production (Stastny, Rosenthal & Ziff, 1975). Thus, the SM in RA may be regarded as an immunologically stimulated lymphoid organ. Two possible explanations can be advanced to explain the chronicicity of this immune activation: antigen may persist or the immunoregulatory mechanisms may be defective. In this latter respect it is relevant that recently, techniques have been established for identifying different subpopulations of T cells in man, which play an important role in immunoregulation (Kung *et al.*, 1980). These T cell subsets may be identified in functional studies or by the presence of specific cell surface markers and are referred to as $OKT4^+$ cells of inducer type and $OKT8^+$ cells of suppressor/cytotoxic type (Reinherz & Schlossman, 1980).

In this study we have attempted to characterize the T cell subsets present in the rheumatoid SM by the application of immunofluorescence techniques in tissue section, and have examined the microenvironment in which these subsets exist in an effort to define the immunoregulatory mechanisms which are active in RA.

MATERIALS AND METHODS

Synovial membranes. Samples of SM were obtained from four normal individuals undergoing menisectomy, and at synovectomy or joint replacement from six patients with osteoarthritis and six patients with classical or definite RA as defined by the American Rheumatism Association. The synovial samples were cut into small pieces, mounted on cork, coated with OCT compound (Miles Laboratories), and snap frozen in 2-Methylbutane (BDH) followed by cooling and storage in liquid nitrogen.

Tissue section analysis. Cryostat sections (6μm thick) were cut at $-25°C$, placed on glass slides, allowed to dry at room temperature for 20 min, following which they were fixed in ethanol at 4°C for 5 min and then washed in phosphate-buffered saline (PBS); pH 7·4) for 10 min. The sections were then incubated with an appropriate volume of diluted antisera for 30 min in a humid chamber at room temperature and finally washed for 10 min in PBS. In the indirect immunofluorescence test the same procedure followed by washing in PBS was used for the second and third layers which were labelled with fluorescein isothiocyanate (FITC, Green) or tetraethyl rhodamine isothiocyanate (TRITC, Red). The sections were mounted in PBS-Glycerol under a cover slip and were examined for fluorescent staining under a Zeiss microscope equipped with an epifluorescence condenser IV/Z and selective filters for FITC and TRITC. Using this technique antibodies could be viewed on the same section simultaneously by simply changing the filters via an interchangeable filter attachment. The indirect immunofluorescence tests were controlled by simultaneously incubating sections with species compatible normal serum, or in the case of mouse monoclonal antibodies, by using ascitic fluid produced in mice by injecting a mouse myeloma cell line.

Antisera. The reagents used are listed in Table 1.

The combinations most often used in this study were:—

(1) OKT3 + anti-Factor VIII related protein
(2) OKT3-B + Chicken anti-HLA-DR
(3) OKT4-B + Chicken anti-HLA-DR
(4) OKT4-B + OKT8-Ars
(5) IgM + OKT4-B
(6) IgM + IgD

These combinations were used on serial sections to analyse the organisation of the cellular

Table 1. Reagents used

	First layer			Second layer
Antibody	Raised in	Reactivity	Reference	Antibody
(A) *Heterologous antisera*				
(1) Human Ig classes (μ, γ, δ)	Goat, Rabbit	B cells; Plasma cells		Directly conjugated reagents (FITC or TRITC)
(2) Factor VIII related protein	Rabbit	Endothelium	Tuddenham *et al.* (1974)	Goat anti-rabbit Ig-FITC
(3) HLA-DR (Ia-like) antigen.	Chicken (against p. 28, 33 antigen)	±Myeloblasts; +Monocytes, B cells; ++Tissue macrophages, veiled cells, Langerhans cells, ID cells	Janossy *et al.* (1980a, 1980b)	Sheep anti-chicken Ig-TRITC
(B) *Monoclonal antibodies*				
(1) OKT3 (Biotin conjugate)	Mouse	Cortical and medullary thymocytes and all peripheral T cells	Kung *et al.* (1979)	Avidin-FITC
(2) OKT4 A and D (Biotin conjugates)	Mouse	T helper/inducer lymphocytes	Kung *et al.* (1980) Reinherz *et al.* (1979) Reinherz & Schlossman (1980)	Avidin-FITC
(3) OKT8A (Arsenilate conjugate)	Mouse	T suppressor/cytotoxic lymphocytes	Kung *et al.* (1980) Reinherz *et al.* (1980)	Rabbit anti-arsenilate-TRITC
(4) OKT6/NA1/34	Mouse	Cortical thymocyte antigen (HTA-1) on 90% cortical and 5% medullary thymocytes; Langerhans cells	Kung *et al.* (1980) McMichael *et al.* (1979)	Goat anti-mouse Ig-FITC

Ig = immunoglobulin; FITC = fluorescein isothiocyanate; TRITC = tetraethyl rhodamine isothiocyanate.

OKT4A and D, and OKT8A have identical reactivity to OKT4 and OKT8 antibodies.

Dilution of antisera used were based on careful standardization of the antisera in tissue section in this laboratory. Monoclonal antibodies were ascitic fluids used in 1:100–1:500 dilution, except NA1/34 a culture supernatant used in 1:10 dilution.

Five to ten microlitres of the appropriate dilution of antisera was used.

infiltrate, and to identify inducer (OKT4$^+$) and suppressor/cytotoxic (OKT8$^+$) subsets. The relationships of these subpopulations to large non-lymphoid strongly HLA-DR$^+$ cells of macrophage lineage, B cells and plasma cells (immunoglobulin positive) were also studied.

RESULTS

Normal and osteoarthritic SM

The analysis of samples obtained from normal individuals and osteoarthritic patients revealed three cell types which expressed HLA-DR antigens (Fig. 1). These were (1) vascular endothelial cells which expressed HLA-DR antigens moderately strongly, although on occasion the endothelium of some vessels appeared negative. (2) Strongly HLA-DR$^+$ cells which morphologically resembled the interdigitating reticulum cells of lymph node. These were distributed around blood vessels and scattered throughout the sublining layer of the SM. (3) Synovial lining cells which also expressed HLA-DR antigens strongly. In the normal SM 20–30% of the lining cells were HLA-DR$^+$ and in the osteoarthritic samples this proportion was increased to around 60%. No lymphocytic infiltrates were observed in any of the samples examined.

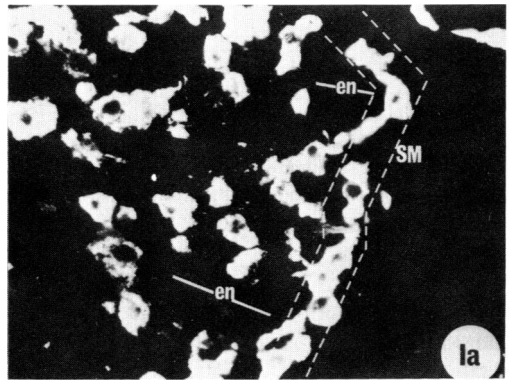

Fig. 1. Osteoarthritic synovial membrane stained with anti HLA-DR (Ia-like) antiserum. Three cell types express HLA-DR antigens: (1) Vascular endothelial cells (weakly positive). (2) Scattered cells in the sub-lining layer which resemble interdigitating cells (strongly positive). (3) A population of synovial lining cells. (SM = synovial membrane lining cells; en = vascular endothelium).

Rheumatoid SM

In all six membranes analysed, the lymphocytic infiltrate was arranged in four characteristic ways. These were (1) scattered diffuse lymphocytic infiltrates, (2) perivascular lymphocytic infiltrates, (3) lymphoid follicles and (4) in one sample only, germinal centres. The immunohistological analysis of these various types of lymphocytic infiltrate will be considered separately.

(1) Scattered diffuse lymphocytic infiltrates

These infiltrates were most prominent beneath the strongly HLA-DR$^+$ hyperplastic synovial lining cells although not confined to this area. They consisted almost entirely of OKT3$^+$ cells (T lymphocytes) which expressed the OKT4$^+$ inducer phenotype with only occasional OKT8$^+$ suppressor/cytotoxic cells being present. Very few IgM/IgD$^+$ B lymphocytes were seen. There did not appear to be a particularly close anatomical relationship between these diffusely scattered lymphocytes and either the HLA-DR$^+$ synovial cells or the large irregularly shaped HLA-DR$^+$ non-lymphoid cells seen in the sub-lining layers.

(2) Perivascular lymphocytic infiltrates (Fig. 2)

Weak to moderately strong expression of HLA-DR antigen was seen on the endothelium of many

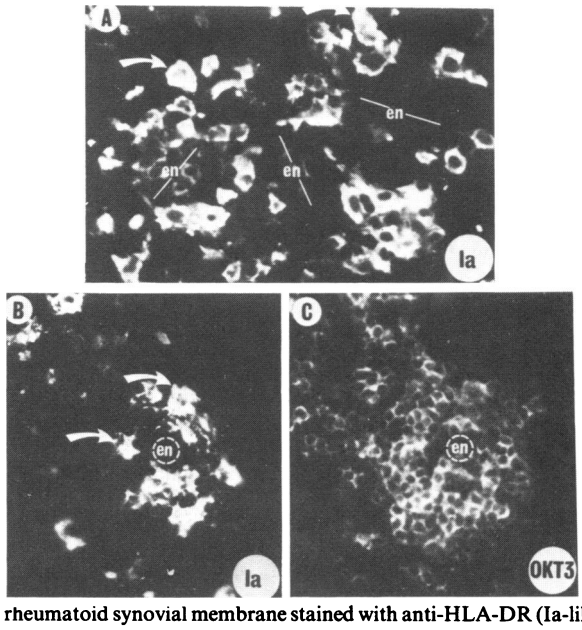

Fig. 2. 2A.A section ot rheumatoid synovial membrane stained with anti-HLA-DR (Ia-like) antiserum, showing weakly HLA-DR$^+$ vascular endothelial cells in the synovial membrane sub-lining layer surrounded by strongly HLA-DR$^+$, non lymphoid, interdigitating cells (arrowed). (en = vascular endothelium) 2B and C. A section of rheumatoid perivascular infiltrate stained simultaneously with antiserum to HLA-DR (Ia-like) antigen and OKT3 antibody. The vascular endothelium is surrounded by strongly HLA-DR$^+$ interdigitating cells (arrowed) and they are surrounded by an OKT3$^+$ lymphocytic infiltrate (T cells). (en = vascular endothelium)

but not all blood vessels, and the blood vessels were frequently surrounded by many strongly HLA-DR$^+$ non-lymphoid cells. On occasion, the endothelial cells appeared as islands between these strongly HLA-DR$^+$ non-lymphoid cells. In other areas additional lymphoid cells, almost exclusively OKT3$^+$ T cells, showed close apposition to these perivascular HLA-DR$^+$ non-lymphoid cells. Further analysis showed that the majority of these cells were of the OKT4$^+$ inducer type (OKT4:OKT8 ratio = 5:1–10:1). B cells expressing membrane staining for IgM and IgD were infrequent ($< 20\%$ of the total lymphocytic infiltrate).

(3) Lymphoid follicles (Figs 3, 4 and 5)

These constituted the largest collections of lymphocytes seen in the synovial membranes and had a complex structure. Analysis with anti-Factor VIII related protein revealed that they were usually formed around four–five endothelial buds which expressed HLA-DR antigen. The intensity of HLA-DR antigen on these endothelial buds was weaker than that seen on the HLA-DR$^+$ non-lymphoid cells and was similar to that seen on endothelium outside the lymphoid follicles. The lymphocytic infiltrate surrounding these endothelial buds was composed, in five out of six cases, of approximately 70–80% OKT3$^+$ cells and 20–30% IgM$^+$/IgD$^+$ B lymphocytes in the majority of cases, although occasionally follicles composed predominantly of B lymphocytes could be found. In one sample, however, the numbers of OKT3$^+$ cells and IgM/IgD lymphocytes were equal in all the follicles examined.

Further analysis of the OKT3$^+$ lymphocytes revealed that they were, again, mainly of the OKT4$^+$ inducer phenotype (Fig. 3). The ratio of OKT4$^+$:OKT8$^+$ cells varied from 7:1 to 15:1 in some areas. Simultaneous staining with anti-IgM and OKT4 revealed that the IgM$^+$ B lymphocytes were scattered throughout the follicles in small clusters and were in close proximity to the OKT4$^+$ cells (Fig. 4). The analysis of adjacent sections showed that the same lymphoid follicles also contained a population of strongly HLA-DR$^+$ non-lymphoid, macrophage-like cells. These had long finger-like processes and morphologically resembled the interdigitating (ID) cells of the T cell

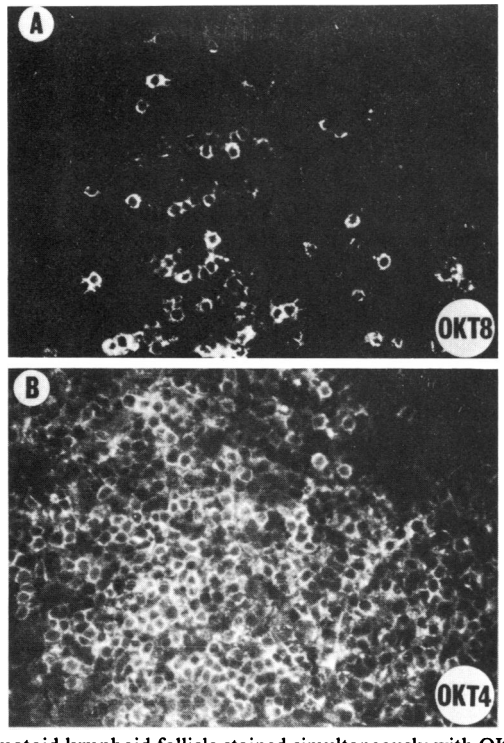

Fig. 3. A section of a rheumatoid lymphoid follicle stained simultaneously with OKT4 and OKT8 antibodies The majority of cells are OKT4$^+$ inducer cells with few OKT8$^+$ suppressor/cytotoxic cells present.

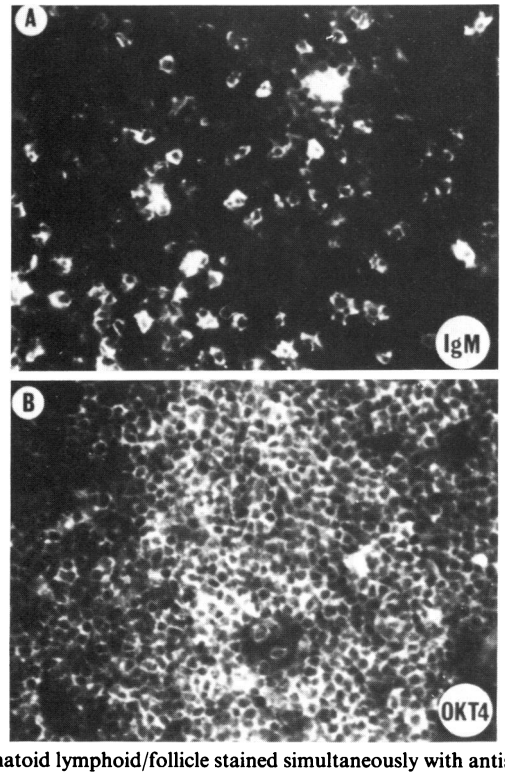

Fig. 4. A section of a rheumatoid lymphoid/follicle stained simultaneously with antiserum to Immunoglobulin M (IgM), Fig. 4A, and OKT4 antibody, Fig. 4B, showing IgM$^+$ B cells scattered throughout the OKT4$^+$ lymphocytic infiltrate.

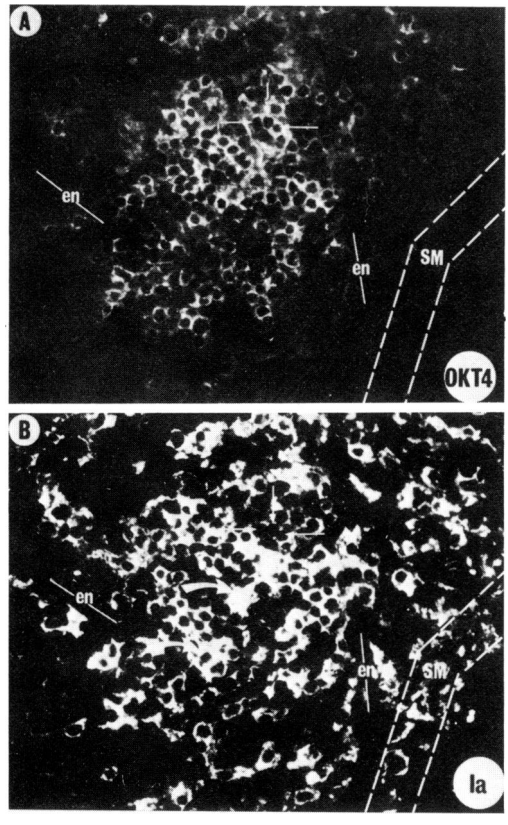

Fig. 5. A section of a small lymphoid follicle beneath the synovial membrane lining layer (SM) stained simultaneously with antiserum to HLA-DR (Ia-like) antigen, Fig. 5B, and OKT4 antibody, Fig. 5A. Strongly HLA-DR$^+$, interdigitating cells (large arrows) are surrounded by OKT4$^+$ inducer lymphocytes. Small arrows indicate OKT4$^+$, inducer lymphocytes which are weakly HLA-DR antigen positive. (en = vascular endothelium).

areas of normal lymph nodes and tonsil. These cells were shown, on analysis with the anti-HLA-DR/OKT4 antisera combination, to be closely surrounded by OKT4$^+$ lymphocytes (Fig. 5). Furthermore, the expression of HLA-DR antigen was observed on the membrane of the majority of lymphocytes present, suggesting that the OKT4$^+$ population, in addition to the B cells and macrophage-like cells, may also express this antigen.

(4) Germinal centres (Fig. 6)

Germinal centres were seen in one sample only. This particular sample had shown large numbers ($\simeq$50%) of B lymphocytes in the lymphocytic infiltrates. The three germinal centres identified in the sample showed the characteristic lacy network of IgM suggesting the deposition of antigen–antibody complexes on putative follicular dendritic cells. In the surrounding lymphocyte corona IgM/IgD$^+$ B lymphocytes were seen. This lymphocyte corona was less well formed than those seen in lymph nodes or tonsil and gave way to a mixed B cell/T cell infiltrate which made up the remainder of the follicle. Some OKT3$^+$/OKT4$^+$ cells of the inducer phenotype were present within the germinal centre.

No positive staining with NA 1/34 antisera to the human thymocyte antigen was seen on any of the HLA-DR$^+$ cells present in the synovial membranes, indicating that cells expressing the phenotype of Langerhans cells were absent in RA.

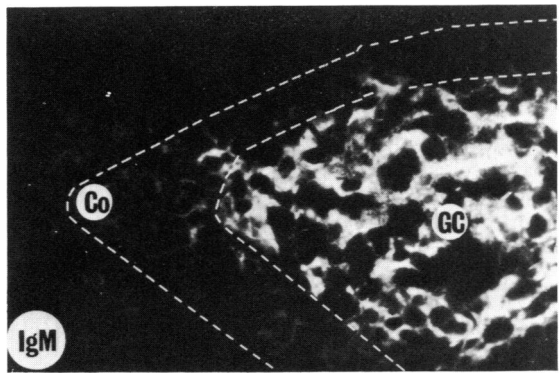

Fig. 6. Section showing a germinal centre (GC) in a rheumatoid synovial membrane surrounded by a poorly formed lymphocyte corona (co) stained with antiserum to immunoglobulin M.

DISCUSSION

The work presented in this paper has been directed towards analysing the nature of the lymphocytic infiltrates, and their microenvironment, in the rheumatoid SM. The results confirm and extend the observations of previous investigators who have shown that the predominant lymphocyte in the rheumatoid SM is the T cell (Froland & Abrahamsen, 1979). These have been shown to be largely cells of the inducer type ($OKT4^+$), with few suppressor/cytotoxic cells present (T4:T8 ratio = 5:1–10:1).

The observation in this study of $OKT4^+$ inducer lymphocytes in clusters around strongly HLA-DR positive ID like cells has been previously reported in thymus (Tidman *et al.*, 1981), normal lymph nodes (LN), tonsil, gut (Janossy *et al.*, 1980b) and in the tissue section of mycosis fungoides (Thomas *et al.*, 1981). This is a further histopathological confirmation of the concept that inducer ($OKT4^+$) cells may see antigens when they are presented to them in association with 'self' HLA-DR antigens (Ia-like antigen) (Dausset & Contu, 1980). In this context it is interesting that the $HLA\text{-}DR^+$, ID-like cells seen in the lymphocytic infiltration of rheumatoid SM are histochemically similar to the ID cells of the paracortical areas of lymph nodes. In addition, the close proximity of the B cells in the lymphoid follicles to T cells of inducer type gives histological evidence of a probable helper interaction for immunoglobulin and RF production in the rheumatoid SM.

The overall appearance of the rheumatoid SM is that of an immunologically stimulated lymphoid organ which bears a close resemblance immunohistologically to the paracortical T cell areas of normal LN. There is however a potentially important difference between the normal LN and the rheumatoid SM. This is that in the former the ratio of inducer ($OKT4^+$) versus cytotoxic/suppressor ($OKT8^+$) cell types is 2:1, while in the rheumatoid SM a numerical deficit of the $OKT8^+$ putative suppressor/cytotoxic population is seen (T4:T8 = 5:1–10:1).

The explanation of these findings is open to debate. We have suggested elsewhere (Janossy *et al.*, 1981) that RA is a disease of T lymphocyte/macrophage immunoregulation in which the HLA-DR positive ID-like cells are able to activate inducer T cells in the absence of effective T cell-mediated suppression. Indeed it has been recently demonstrated that the HLA-DR positive cells from the rheumatoid SM are potent stimulators of T lymphocytes in a mixed lymphocyte reaction (Klaresgog *et al.*, 1981). This positive regulatory influence may lead to a self-perpetuating vicious circle, that would result, in turn, in B lymphocyte activation, further accumulation of macrophage-like cells, and joint destruction. In exceptional cases typical germinal centres could be formed, as was observed in one of our samples.

A number of interesting questions remain to be answered. Firstly, a similar immunohistological analysis is needed of the SM of patients with early RA, as well as in advanced forms of the disease, and to compare these findings with those obtained in patients with seronegative arthropathies

(psoriasis, ankylosing spondylitis, Reiter's disease, etc.). Secondly, functional studies of the lymphocytes present in the rheumatoid SM are also necessary to substantiate the findings suggested by the phenotypic analysis of the T cell subsets. In this respect it is relevant that Chattopadhyay *et al.*, (1979a, 1979b) have recently demonstrated a lack of suppressor cell activity in rheumatoid SM.

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