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A combined immunohistological and histochemical analysis of lymphocyte and macrophage subpopulations in the rheumatoid nodule

O. L. DUKE,† S. HOBBS,* G. S. PANAYI,† L. W. POULTER,* J. J. RASKER‡ & G. JANOSSY* * *Department of Immunology, Royal Free Hospital School of Medicine;*
† *Departments of Medicine and Rheumatology, Guy's Hospital Medical School, London, UK and*
‡ *Department of Rheumatology, Ziekenhuis Ziekenzorg, Enschede, The Netherlands*

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SUMMARY

The histochemical demonstration of acid phosphatase (ACP) and adenosine triphosphatase (ATP) has been combined with standard immunofluorescence techniques, using a panel of monoclonal and conventional antibodies, to examine lymphocyte and macrophage subsets and their microanatomical relationships within the subcutaneous rheumatoid nodule (RN). This analysis reveals that the RN is composed largely of strongly HLA-DR$^+$, ATP$^-$ macrophages which contain lysosomal enzymes (ACP) in large amounts. The lymphocytic infiltrate which is sparse and poorly organized is comprised almost entirely of thymus derived lymphocytes (T cells) with a normal proportion of helper/inducer (OKT4$^+$) and suppressor/cytotoxic (OKT8$^+$) cells. These observations are in contrast to the findings in the rheumatoid synovial membrane of a prevalence of interdigitating type, HLA-DR$^+$ cells and the predominance of helper (OKT4$^+$) type T cells.

Keywords rheumatoid nodule immunohistology macrophage subpopulations

INTRODUCTION

Rheumatoid arthritis (RA) is a disease which is considered to be the result of an abnormal immunological reactivity. This is supported by the presence of hypergammaglobulinaemia, the production of rheumatoid factors (RF) and the characteristic mononuclear cell infiltrate seen within the synovial membrane (SM). Although diarthroidal joints bear the brunt of the attack a proportion of patients develop the extra-articular features of rheumatoid disease, the best known of which is the subcutaneous rheumatoid nodule (RN) occurring in approximately 20% of patients (Gardner, 1972). Histologically the RN is a granuloma composed of a necrotic central area surrounded by radially arranged histiocytes in a characteristic fashion. The periphery of the nodule is composed of loose connective tissue and collections of lymphocytes. Similar lesions may also be seen in other organs of patients with severe RA.

The aetiology of the RN remains uncertain but as it is commonly seen in a clinical setting of RA accompanied by high titres of RF, circulating immune complexes, clinical vasculitis and other manifestations of extra-articular disease, it has been considered to be the result of a local vasculitis resulting from immune complex deposition (reviewed by Gardner, 1972).

The development of immunofluorescent and histochemical techniques to identify T cell and

Correspondence: Professor G. S. Panayi, Department of Rheumatology, Guy's Hospital Medical School, London SE1 9RT, UK.

macrophage subsets in tissue section (reviewed by Poulter *et al.*, 1983a) has allowed the identification of these subsets and analysis of their interactions in normal and pathological tissues. Such an approach may provide clues to the pathogenesis of immunoregulatory disorders. We have undertaken such an analysis of the RN with the view that comparative observations between the RN and SM may provide some insight into the pathogenetic factors contributing to this chronic disease.

MATERIALS AND METHODS

Twelve RN ranging in age from 3 days to 5 weeks were obtained from patients with definite or classical RA. The nodules were cut into small pieces of approximately 5–10 mm diameter, frozen in isopentane cooled in liquid nitrogen, and then stored at −70°C until used.

Immunofluorescence. Cryostat sections (6 μm thick) were cut, air dried at room temperature for 30 min, and fixed for 5 min in a chloroform/acetone mixture (1 : 1) at 4°C and allowed to dry again. The sections were then freeze dried and stored at −20°C until required.

The techniques used for marking with antibodies in direct and indirect immunofluorescent tests have been described previously (Duke *et al.*, 1982). Briefly, the freeze dried sections were washed in phosphate-buffered saline (PBS, pH 7·4) for 10 min prior to addition of the first layer reagents. They were then allowed to incubate for 30 min at room temperature, in a humid chamber, following which they were washed in PBS for 15 min. In the indirect immunofluorescent tests this procedure was repeated for the fluorochrome labelled second and third layer reagents where appropriate. Finally the sections were mounted in glycerol with a coverslip and viewed under a Zeiss microscope equipped with an epifluorescence condenser and selective filters for viewing fluorescein isothiocyanate (FITC, green) and tetraethyl rhodamine isothiocyanate (TRITC, red).

The antisera employed in this study are shown in Table 1. These antibodies were used in combination to allow significant double marker analysis of the sections.

Histochemistry. Acid phosphatase (ACP) and adenosine triphosphatase (ATP) activity were demonstrated on cryostat sections using established techniques (Lojda, Grossman & Schneider,

Table 1. Reagents*

Antibody	Raised in	Reactivity
Heterologous antisera		
Human Ig classes (μ, δ, γ)	Goat, rabbit	B cells; Plasma cells
HLA-DR (Ia like) antigen	Chicken	±myeloblasts; + monocytes, B cells; + + tissue macrophages, veiled cells, Langerhans cells, interdigitating cells. Endothelial cells.
Monoclonal antibodies		
OKT3 (Biotin conjugate)	Mouse	Cortical and medullary thymocytes and all peripheral T cells.
OKT4 (Biotin conjugate)	Mouse	T helper/inducer lymphocytes.
OKT8A (Arsenilate) conjugate)	Mouse	T suppressor/cytotoxic lymphocytes.
OKT6/NA1/34	Mouse	Cortical thymocyte antigen [HTA1) on 90% cortical and 5% medullary thymocytes; Langerhans cells.
FMC-17†	Mouse	Monocytes/macrophages

* References to development and characterization of antisera used are given in Duke *et al.* (1982) as are details of the second layer antibodies.

† Reference—Brooks *et al.* (1983).

1979). Histochemical reactions were also performed on sections previously incubated with fluorochrome labelled antisera. This allowed the simultaneous analysis of enzyme and immunological phenotypes of individual cells. The techniques involved in this combined analysis have been described elsewhere (Poulter *et al.*, 1983b).

RESULTS

Histology

Haematoxylin & eosin stained sections of each nodule were prepared and the histology reviewed. All the nodules showed the classical histological appearances of RN (vide supra) although variations were noted between nodules in the following features. Firstly, young nodules usually contained a single necrotic area surrounded by radically arranged histiocytes whilst older nodules often contained two or three such areas. Secondly, whilst a few diffusely scattered lymphocytes were seen in the periphery of all nodules the extent of the perivascular lymphocyte infiltrate was highly variable. Three of the 12 nodules examined showed well formed perivascular lymphoid follicles whilst in the remaining nodules very few perivascular lymphocytes were observed. Finally, no clear correlation existed between the age of the nodule and the degree of lymphocytic infiltration.

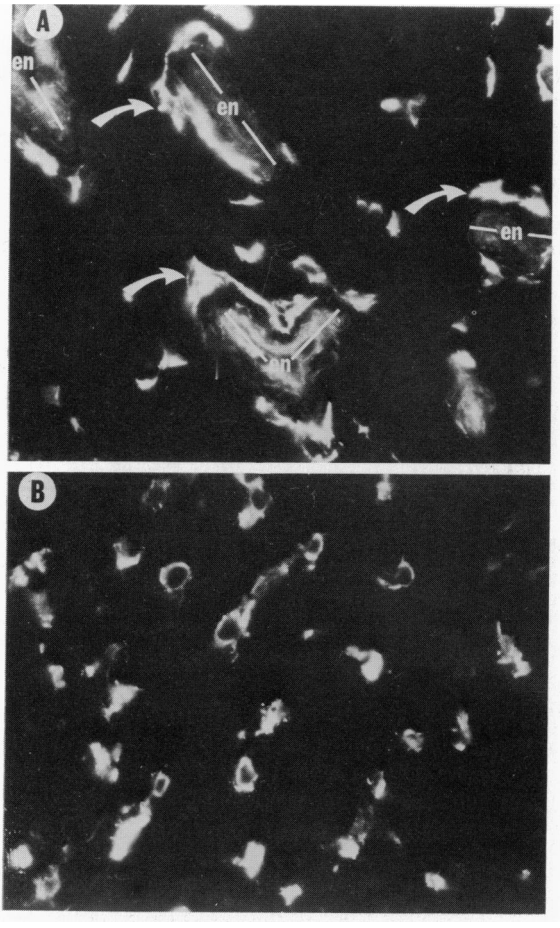

Fig. 1. (A) A section of the periphery of a RN stained with anti-HLA-DR (Ia like) antiserum, showing weakly HLA-DR$^+$ vascular endothelium (en) surrounded by strongly HLA-DR$^+$ macrophages (arrowed) which are scattered throughout the surrounding connective tissue. (B) A section through the palisade layer of a RN showing that these macrophages are also HLA-DR$^+$.

Immunofluorescence and histochemistry

Distribution of HLA-DR antigen. In the periphery of the nodules HLA-DR antigen was seen weakly on vascular endothelium and very strongly on large, irregularly shaped, non-lymphoid cells. The latter were distributed around blood vessels and scattered throughout the periphery of the nodules (Fig. 1A) in a pattern similar to that seen in some areas of normal and osteoarthritic synovial membranes. These cell types have also been observed in rheumatoid synoival membrane where they are present in much greater numbers than in the RN (Duke *et al.*, 1982). In the centre of the nodule the palisading histiocytes surrounding the necrotic areas were also HLA-DR positive (Fig. 1B).

Lymphocyte subsets. Ninety-five to one hundred per cent of lymphocytes comprizing the perivascular and diffusely scattered infiltrates were OKT3$^+$ cells (T cells), and in the few perivascular accumulations observed the use of the OKT3/HLA-DR antibody combination revealed a close microanatomical relationship between these cells and large, HLA-DR$^+$, non-lymphoid cells; furthermore, between 0–30% of the OKT3$^+$ cells in these perivascular sites expressed HLA-DR antigens (Fig. 2).

In order to examine the immunoregulatory mechanisms operating within the lymphocytic infiltrates of the RN a phenotypic analysis of the T cell subsets was performed using the OKT4 and OKT8 monoclonal antibodies (see Table 1). This disclosed the presence of both OKT4$^+$ (inducer)

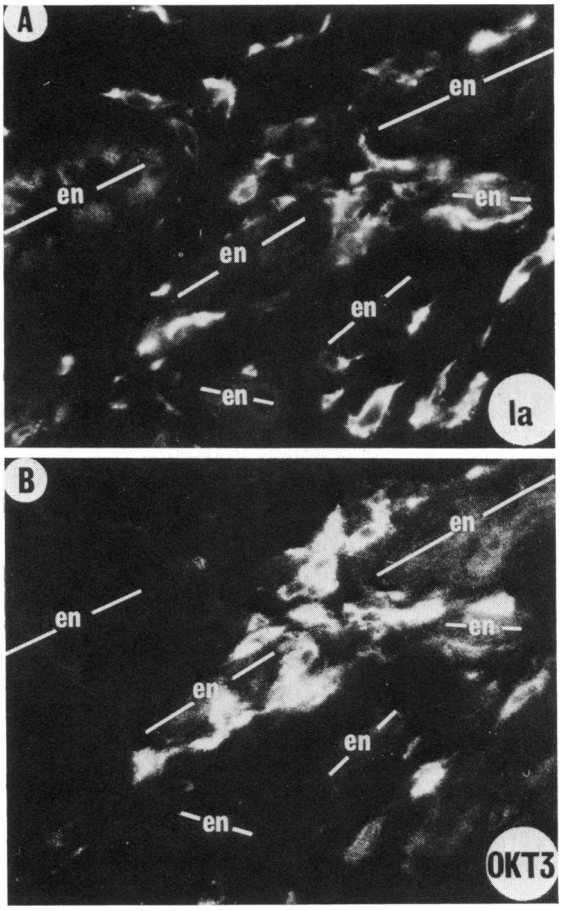

Fig. 2. A section through a small perivascular infiltrate of lymphocytes in the periphery of a RN stained with anti-HLA-DR (Ia like) antiserum (A) and OKT3 antibody (B). None of the OKT3$^+$ lymphocytes appear to express HLA-DR antigens in this section (en = endothelium).

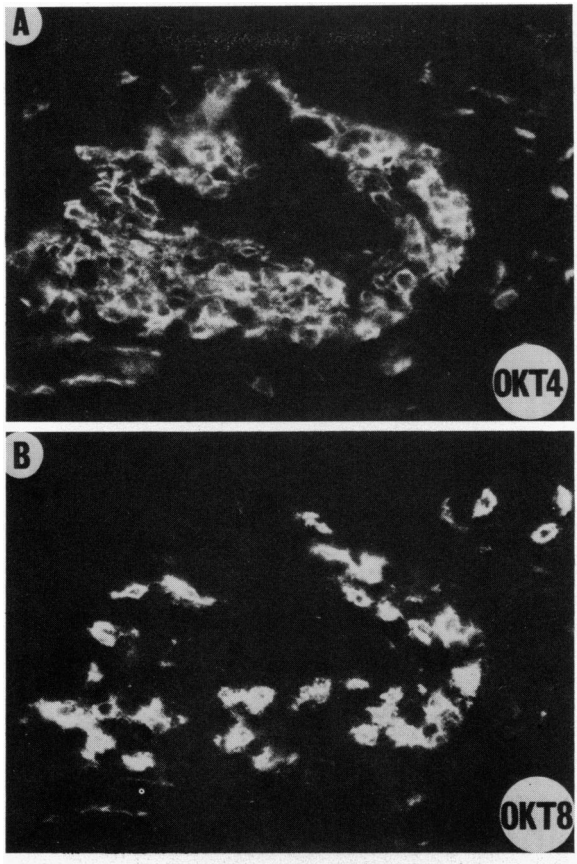

Fig. 3. A section illustrating one of the largest perivascular accumulation of lymphocytes seen in our series of RN. Stained with OKT4 antibody (A) and OKT8 antibody (B). This reveals a normal ratio of OKT4$^+$, inducer cells, to OKT8$^+$, suppressor/cytotoxic cells, of approximately 2–3:1.

cells and OKT8$^+$ (suppressor/cytotoxic cells in ratios varying from 1:1–3:1 (OKT4:OKT8; Fig. 3); such ratios approximate those found in the peripheral blood and T cell areas of lymph nodes of normal individuals (Janossy *et al.*, 1980).

The B cells were sparse or absent never exceeding 5% of the total lymphocytic infiltrate. No plasma cells or germinal centres were seen.

Macrophage subsets. Simultaneous analysis with FMC-17, a monocyte/macrophage marker, and anti-HLA-DR antiserum on the same section revealed that $\simeq$85–95% of the large, HLA-DR$^+$, non-lymphoid cells present in the periphery and in the central palisading layer of the RN were FMC-17$^+$. Some cells ($\simeq$5–15%) were seen which expressed either the FMC-17 marker or HLA-DR antigen but not both. These findings were remarkably constant from one nodule to another and the FMC-17$^+$ cells appeared to be the most frequent cell type in the RN. An attempt to further characterize the phenotype of the FMC-17$^+$ cells using the NA1/34 antibody revealed that this reagent, which recognises immature thymocytes and Langerhans cells, failed to react with any of the HLA-DR$^+$/FMC-17$^+$ cells. Characterisation of the FMC-17 antibody (Seymour *et al.*, 1983) has shown that it reacts with two different subsets of strongly HLA-DR$^+$ macrophage like cells. The first is ATPase positive and contains only small amounts of ACP (interdigitating or dendritic cell) whilst the second one can be considered as an activated macrophage with a high level of ACP and little or no ATPase activity. Thus in order to establish the identity of the FMC-17$^+$HLA-DR$^+$ cells in the RN a combined histochemical (ACP or ATPase) and immunofluorescent (FMC-17 or HLA-DR) analysis was performed. The results (summarized in Table 2) revealed that in the

Table 2. Macrophage subsets and their distribution within the RN

Cell type	Phenotype				Location in RN	
	HLA-DR	FMC-17	ACP	ATPase	Periphery (perivascular & scattered)	Central (palisade layer)
Tissue macrophages	−	+	+	−	1–5%	<1%
Activated macrophages	+	+	+	−	85–95%	95–99%
Cells of interdigitating type	++	+	−	+	1–10%	1–5%

FMC-17 is a monocyte/macrophage specific monoclonal antibody.

periphery of the RN approximately 90% of the FMC-17$^+$ macrophages were strongly positive for the enzyme ACP (Fig. 4) and negative or weakly positive for ATPase. In addition a smaller population of macrophages was observed which expressed ATPase activity strongly and were only weakly ACP positive; this cell type accounted for < 10% of the FMC-17$^+$ macrophages. These two populations of macrophages appeared to be similarly distributed and were observed in the

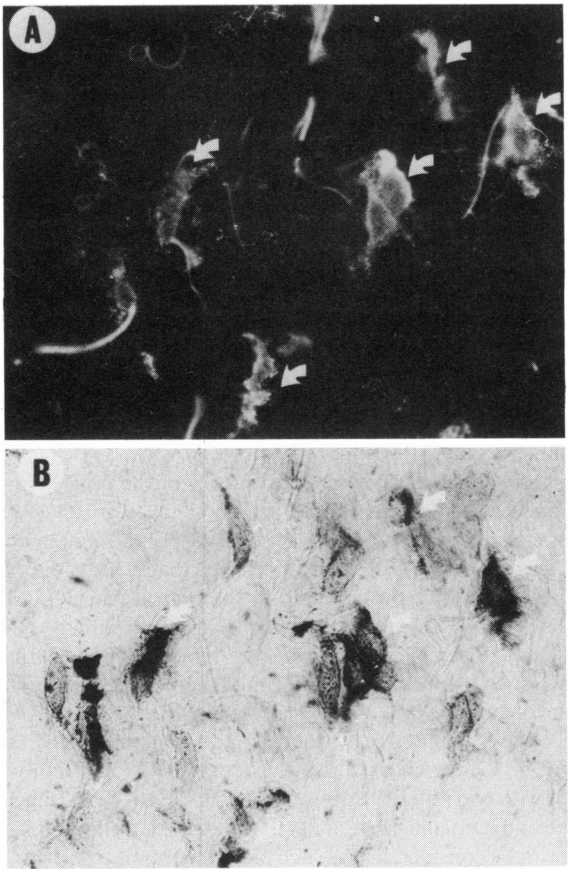

Fig. 4. A section through a RN stained with the FMC-17 antibody, (A) and simultaneously histochemically stained to show acid phosphatase (ACP) activity (B). The majority of the FMC-17 macrophages are ACP$^+$. Corresponding cells in (A) and (B) are marked with arrows.

perivascular areas as well as being diffusely scattered in the periphery of the RN. In the centre of the RN the palisading layer of cells were composed of HLA-DR$^+$/FMC-17$^+$ macrophages which showed strong ACP activity with $< 5\%$ expressing strong ATPase activity.

DISCUSSION

Our study has concentrated on the identification of lymphocyte and macrophage subsets and their inter-relationships in a series of RNs ranging in age from 3 days to 5 weeks old. The results have revealed two important features of the RN which are, firstly, that the predominant macrophage subset present in these lesions expresses large amounts of HLA-DR antigen and is strongly positive for the lysosomal enzyme ACP and, secondly, the lymphocytic infiltrate is composed of both helper and suppressor/cytotoxic thymus derived lymphocytes (T lymphocytes) in a ratio of 1 : 1–3 : 1. These findings are of particular interest when compared with the observations made in similar studies of the rheumatoid SM and delayed type hypersensitivity (DTH) reactions (Duke *et al.*, 1982; Poulter *et al.*, 1982, 1983b) as they serve to focus attention on the possible immunopathological events which may be operating in RA.

The macrophage population within the RN is similar to that seen in a resolving DTH reaction being composed predominantly ($\simeq 95\%$) of cells which express HLA-DR antigens strongly and, in addition, have strong lysosomal enzyme (ACP) activity. This latter feature is a characteristic of cells of the monocyte/macrophage series and particularly of activated inflammatory macrophages (van Furth *et al.*, 1975; Poulter & Turk, 1975). Furthermore, whereas in the bone marrow and normal lymphoid tissues these ACP positive macrophages usually express only small amounts of HLA-DR antigens (Seymour *et al.*, 1983), in the RN they characteristically express large amounts of HLA-DR antigen and are therefore best regarded as activated macrophages. However, HLA-DR antigens are thought to be important in mediating the cellular interactions necessary for antigen presentation (Dausset & Contu, 1980) and are present on a variety of cell types (including B lymphocytes, monocytes, activated macrophages, interdigitating cells and Langerhans cells) which are known to be able to function as antigen presenting cells (Balfour *et al.*, 1981; Humphrey, 1981). Thus it is possible that the activated macrophages seen in the RN may be capable of functioning as antigen presenting cells as well as fulfilling a degradative or 'scavenger' role. They therefore contrast with the interdigitating cells (IDC) which have very large amounts of HLA-DR antigens but little or no ACP activity and are regarded as the most highly specialized antigen presenting cell with no degradative function (Balfour *et al.*, 1981). It is important in this context that while the rheumatoid SM contains many activated macrophages it also contains a large population ($\simeq 80\%$ of stromal macrophages) of strongly HLA-DR positive cells with dendritic morphology and strong ATPase activity but only weak lysosomal enzyme activity (Poulter *et al.*, 1982). These features are characteristic of IDC in normal lymphoid tissue (Muller-Hermelink, 1974). Such HLA-DR positive cells form $< 10\%$ of the macrophages seen in the RN. Furthermore in the rheumatoid SM these IDC are present in a close microanatomical relationship to many helper (OKT4$^+$) T lymphocytes (Duke *et al.*, 1982) and may contribute to a mutually stimulatory interaction between themselves and the helper lymphocytes as previously suggested (Janossy *et al.*, 1981). The important finding of this paper is that this situation does not appear to exist within the RN. These microenvironmental differences may explain the chronic persistent nature of the inflammatory infiltrate in the SM whereas the RN is inclined to be a more evanescent and benign manifestation of the disease.

The other observation of interest was that the sparse lymphocytic infiltrate seen in the RN was composed of both helper and suppressor/cytotoxic T lymphocytes in a ratio similar to that seen in the T cell areas of normal lymphoid tissues and in DTH reactions. This suggests that normal immunoregulatory mechanisms exist in both the RN and in the DTH reaction in contrast to the situation in the rheumatoid SM where the excess of helper T lymphocytes may contribute to a pathological overactivation.

It is clear therefore that although the events leading to the formation of the RN, and in particular the role of immune complex-induced vasculitis, remain uncertain (Rasker & Kuipers, 1983) the immunological response differs from that seen in the rheumatoid SM. In the RN the

reaction resembles a resolving DTH reaction with activated macrophages whereas a more permanent immunoregulatory disorder with many putative antigen presenting cells develops in the SM. These findings highlight the importance of differential homing and functional characteristics of the various macrophage subsets in RA and it will be important to investigate the roles of activated tissue macrophages and IDC in maintaining the chronicity of the disease by means of tissue culture experiments as recently suggested (Winchester & Burmester, 1981; Klaresgog *et al.*, 1982).

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