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The distribution and functional properties of dendritic cells in patients with seronegative arthritis

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SUMMARY

Dendritic cells (DC), potent antigen-presenting cells, are known to be increased in numbers in inflammatory lesions in rheumatoid arthritis and juvenile chronic arthritis. In this study, patients with seronegative arthritis were studied; the distribution and functional properties of DC enriched low density cells (LDC) from peripheral blood (PB) and synovial fluid (SF) were compared. The composition of LDC from both sources was similar, comprising approximately 30% DC, 60% monocytes with few T lymphocytes. SF was significantly enriched for LDC compared with paired peripheral blood ($P < 0{\cdot}0001$) or peripheral blood from healthy controls ($P < 0{\cdot}001$). In contrast, patient PB contained fewer LDC ($P < 0{\cdot}05$) overall than healthy controls. LDC from both sources were potent stimulators of allogeneic PB T cells in a mixed leucocyte reaction (MLR), but in four out of 10 patients SF LDC were significantly more stimulatory. In autologous MLRs (AMLRs) SF T cells were not stimulated by either LDC population. This anergy of T cells was confined to the joint as patient PB T cells showed an AMLR response to PB LDC which was similar to that seen in cells from healthy controls. PB T cells also responded to SF LDC; in a minority of patients SF LDC caused significantly greater stimulation in AMLR than PB LDC and the possibility is discussed that this may represent presentation of antigen acquired *in vivo*.

Keywords dendritic cells arthritis autologous mixed leucocyte reaction

INTRODUCTION

In genetically susceptible individuals infection of the gastrointestinal or genital tracts with a number of Gram-negative bacteria can give rise to arthritis. This condition, known as reactive arthritis, is characterized by a failure to isolate viable organisms from the affected joints (reviewed by Keat, 1983). However, recent evidence suggests that antigenic material derived from the triggering organism does localize to the joint: antigens of *Chlamydia trachomatis* (Schumacher *et al.*, 1986; Keat *et al.*, 1989) and *Yersinia entercolitica* (Granfors *et al.*, 1989) have been detected by immunofluorescence with appropriate monoclonal antibodies. T cells responsive to the triggering antigen have also been detected in the joints of these patients (Ford *et al.*, 1980; Gaston *et al.*, 1989), strengthening the suggestion that a localized immune response directed against bacterial products may be an underlying cause of the arthritis.

Bone marrow-derived dendritic cells (DC) have been shown to be very potent antigen-presenting cells *in vitro* (reviewed by Austyn, 1987). In addition, DC are important in development of delayed-type hypersensitivity reactions (Macatonia, Edwards & Knight, 1986) and induction of autoimmunity (Knight *et al.*, 1986) *in vivo*. In rheumatoid arthritis (RA), cells with phenotypic characteristics of DC have been described in the synovial membrane in physical association with $CD4^+$ T cells, and large numbers have been isolated from synovial fluids (Tyndall, Knight & Edwards, 1983; Harding & Knight, 1986). It has been suggested that these cells may be presenting an as yet unidentified antigen to T cells within the joint resulting in T cell activation, recruitment of inflammatory cells and subsequent joint damage (Knight, 1988). Further studies in this area have been difficult since the identity of this putative antigen is unknown in juvenile chronic arthritis (JCA) and RA. We have therefore turned to patients with reactive arthritis where candidate antigens, at least at the whole organism level, have been identified. Here we report the isolation and preliminary characterization of DC from patients with reactive and other arthropathies and investigate the possibility that cells from the joint are presenting antigen.

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MATERIALS AND METHODS

Patients

Sixty-one patients were included in the study of whom 21 had a seronegative lower limb oligo- or monoarthritis and evidence of

genital or gastrointestinal tract infection. Of these, 17 had a clinically proven urethritis or cervicitis within the preceding 3 months or contemporaneous with the arthritis and these patients were regarded as having sexually acquired reactive arthritis (SARA). Three patients had had significant diarrhoea within the 3 months preceding the onset of arthritis and these were regarded as having enteropathic reactive arthritis (ERA). A single patient fulfilled the criteria for both SARA and ERA and was classified as SARA/ERA. A further 20 patients with seronegative disease showed no clear evidence of infection and these were classified as undifferentiated seronegative arthritis (USA). In addition, 16 patients with other inflammatory arthropathies (RA, psoriatics) and four patients with osteoarthritis were included. Normal controls were healthy laboratory personnel.

Isolation of low density cells (LDC)

Mononuclear cells were isolated from defibrinated peripheral blood or synovial fluid collected in acid–citrate–dextrose (ACD) by centrifugation over Ficoll–Paque (Pharmacia Fine Chemicals) (600 ***g***, 30 min). Highly viscous fluids were treated with 10 U/ml hyaluronidase (Hyalase) before centrifugation. Neither Hyalase nor the use of ACD as anticoagulant was found to affect lymphocyte stimulation in the assays used. Washed cells were cultured overnight in complete medium (Dutch modification of RPMI 1640 containing 10% fetal calf serum (FCS), 100 μg/ml L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin). Cells were cultured at 5×10^6/ml in 25-cm^2 tissue culture flasks (Falcon Co., Cockeysville, MD). After overnight incubation (37°C, 5% CO_2 in air) non-adherent cells were spun over 14·25% w/v metrizamide at 600 ***g*** for 10 min. Low density cells, enriched for DC, were collected from the interface and washed twice. Adherent cells from the overnight culture were recovered with a rubber policeman and used as a source of macrophages. Pellet cells from the metrizamide gradient were enriched for T cells by rosetting with 2-amino ethyl isothiouronium bromide (AET)-treated sheep erythrocytes (Saxon, Feldhaus & Robins, 1976). Rosetted cells (E^+; T cells) and non-rosetting cells (E^-; rich in B cells) were separated by centrifugation over Ficoll–Paque (600 ***g***, 15 min). Sheep erythrocytes were lysed with Gey's haemolytic solution.

Phenotypic analysis

Phenotypic analysis LDC were labelled in 0·1 ml phosphate-buffered saline (PBS) containing 2·5% FCS, 100 mM EDTA, 0·02% azide with optimal concentration of one of the following monoclonal antibodies: Leu M3 (anti-CD14, Becton Dickinson), RFD1 (anti-HLA-D region product displaying preferential reactivity with DC, a gift from Dr L. Poulter) or UCHT1 (anti-CD3). Cells were incubated on ice for 30 min, washed twice in labelling buffer and incubated with fluorescein-labelled anti-mouse immunoglobulin (Dako, High Wycombe, UK) at a dilution of 1 in 20 for 30 min on ice. The cells were washed twice and then examined by fluorescence microscopy (Zeiss, Obercochen, Germany) and the percentage labelled determined.

Cell cultures

Triplicate cultures were established in inverted 60-well Terasaki plates (Falcon). A total of 40000 or 50000 pellet lymphocytes or purified T cells was added per well. For allogeneic mixed leucocyte reaction (MLR), lymphocytes were isolated from the peripheral blood of healthy individuals chosen at random. Stimulator DC were irradiated (20 Gy) and added at 100, 300, 1000 or 3000 cells per well. The plates were inverted over saline in a plastic box in a humidified incubator gassed with 5% CO_2. Tritiated (^{3}H) thymidine (New England Nuclear, Cambridge, MA; 1 μl giving 1 μg/ml of thymidine at specific activity of 2 Ci/mmol) was added 2 h before harvesting on day 5 using a hanging drop blot technique (Knight, 1987b).

Statistical analysis

^{3}H-thymidine uptake from triplicate cultures of peripheral blood (PB) and synovial fluid (SF) lymphocytes was determined by standard liquid scintillation counting techniques. Differences between paired stimulator cell populations were tested for significance across a concentration range using an analysis of variance method (Knight *et al.*, 1981). Maximum stimulation levels were compared using a *t*-test.

RESULTS

Isolation of LDC from PB and SF

Figure 1 shows the number of LDC isolated from 60 patients with a variety of arthropathies and 25 healthy controls. The results were expressed as LDC as a percentage of total mononuclear cells obtained in order to allow for differences in cellularity between blood and synovial fluid. Overall, patient PB mononuclear cells contained between 0·43 and 13% LDC, significantly ($P < 0·05$) fewer than healthy controls (11–26%) although when the three major patient groups (SARA/ERA, USA, and other joint disease) were considered separately, only for SARA/ERA patients did the difference reach statistical significance. In contrast, synovial fluids were significantly enriched for LDC (3·0–56·0%) compared with paired blood samples ($P < 0·0001$) or, overall, with peripheral bloods of healthy controls ($P < 0·001$). There did not appear to be any difference between the three major patient groups with regard to LDC numbers in PB or SF. This includes the patients with osteoarthritis but it should be noted that the patient from whom

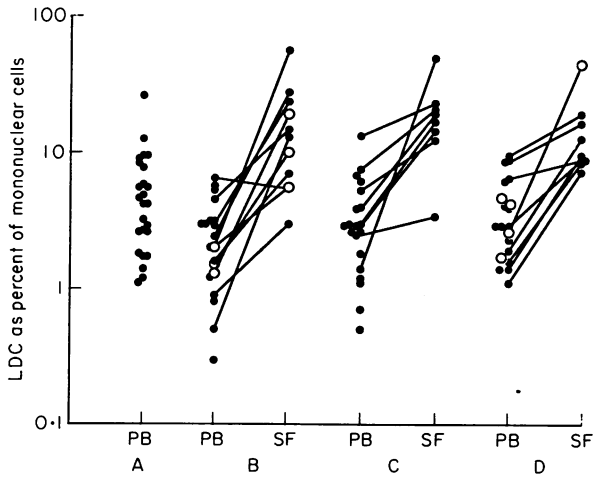

Fig. 1. Isolation of low density cells (LDC) from peripheral blood (PB) and synovial fluid (SF). Results are expressed as per cent of total mononuclear cells. (A) Healthy controls; (B) SARA (●) or ERA (○); (C) USA; (D) other arthopathies: inflammatory (●) or non-inflammatory (○). For definitions of arthritis types see Materials and methods.

Table 1. Phenotypic characterization of peripheral blood (PB) and synovial fluid (SF) low density cells (LDC)

Antibody	Specificity	Per cent of LDC positive		
		Control PB	Patient PB	Patient SF
Leu M3	CD14 monocytes	63·7 (32–79) $n=14$	62·0 (32–91) $n=16$	46·1 (6–70) $n=8$
RFD1μ	HLA-D region product Dendritic cells	35·2 (7–72) $n=14$	33·3 (0–77) $n=16$	34·1 (5–67) $n=8$
UCHT1	CD3 T cells	10·9 (0–28) $n=12$	8·1 (0–26) $n=15$	17·5 (3–47) $n=8$

Results are means (range).

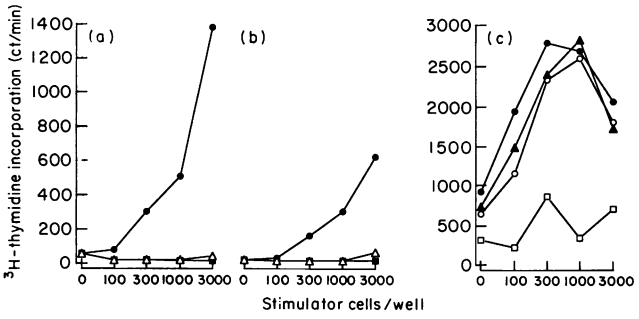

Fig. 2. CD14$^-$ low density cells (LDC) are the major stimulators of the mixed leucocyte reaction (MLR). (a) Expt 1. Stimulation of peripheral blood (PB) lymphocytes by allogeneic LDC (●), adherent macrophages (△) and E$^-$ cells (■). (b) Expt 1. Stimulation of PB lymphocytes by autologous LDC (●), adherent macrophages (△) and E$^-$ cells (■). (c) Expt 2. Stimulation of allogeneic PB lymphocytes by untreated LDC (●), LDC treated with complement alone (○), Leu M3 plus complement (▲) or RFD1μ plus complement (□).

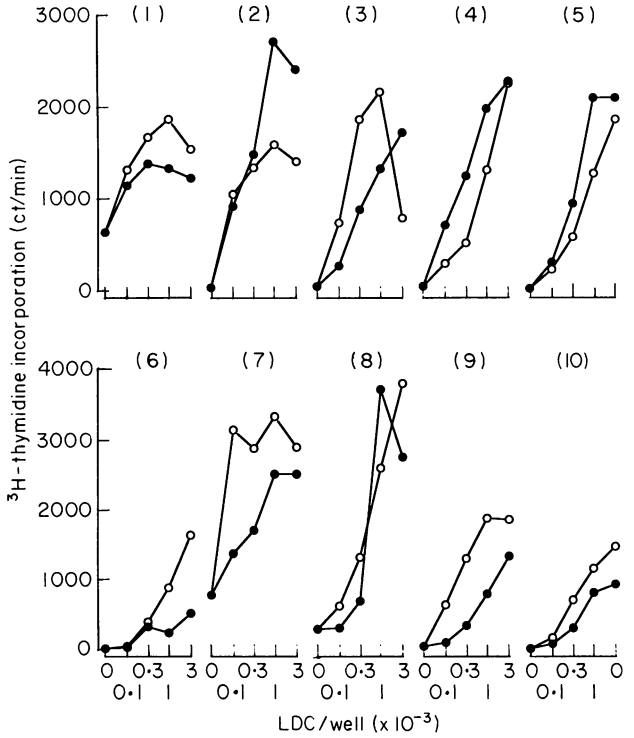

Fig. 3. Stimulation of allogeneic peripheral blood (PB) T cells from healthy controls by PB (●) and synovial fluid (SF) (○) low density cells (LDC) isolated from 10 patients with inflammatory arthritis. Patients 1–3: SARA; patient 4: SARA/ERA; patients 5–8: USA; patient 9: RA; patient 10: psoriasis. For definitions of arthritis types see Materials and methods.

SF was obtained was unusual for individuals with 'non-inflammatory' conditions in that the fluid was highly cellular, making this kind of analysis possible.

Phenotypic characterization of PB and SF LDC

The cellular composition of LDC from the SF of eight patients (five SARA, two USA, one RA) was compared with LDC from patient and control PB. The composition of all three populations was broadly similar (Table 1). Approximately one-third of the cells labelled with RFD1, an antibody which selectively labels DC (Poulter *et al.*, 1982), whereas twice as many cells labelled with an antibody to the monocyte marker CD14. By immunogold electron microscopy there is very little double labelling with RFD1 and anti-CD14 (Knight *et al.*, 1989). LDC isolated from peripheral bloods generally contained few T lymphocytes. Slightly elevated numbers were found in SF LDC, possibly as a consequence of the presence of lymphoblasts in the joints.

CD14$^-$ LDC are the major stimulators of the MLR

In preliminary experiments, LDC, adherent macrophages and B cell-rich E$^-$ cells were isolated from control peripheral blood. Only LDC were able to stimulate a strong MLR (Fig. 2a) or autologous MLR (Fig. 2b). Removal of CD14$^+$ monocytes from the LDC population by antibody and complement treatment had no effect on the ability to stimulate MLR. In contrast, LDC treated with RFD1 and complement were very poor stimulators. Thus, the active cell population in this assay is likely to be a RFD1$^+$ DC (Fig. 2c).

Stimulation of MLRs by PB and SF LDC from patients with arthritis

Both PB and SF dendritic cells from arthritis patients are potent stimulators of the allogeneic MLR (Fig. 3). Addition of as few as 2·5% LDC was sufficient to cause strong stimulation in all cases.

Overall, when maximum levels of allostimulation by the two populations were compared there was no significant difference ($P=0·52$; non-significant, data not shown). When paired samples from individual patients were compared across a cell concentration range no statistically significant differences between PB and SF LDC were observed in six out of 10 patients including all of those with reactive arthritis ($P>0·05$). In four out of 10 patients (patients 6 (USA), 7 (USA), 9 (RA), 10 (psoriatic)) SF LDC were significantly more stimulatory than PB LDC ($P<0·05$). This was particularly striking in patient 7 (USA) where 100 SF LDC were more stimulatory than 3000 PB LDC. In no case where a higher response was elicited by PB LDC was this difference found to be statistically significant.

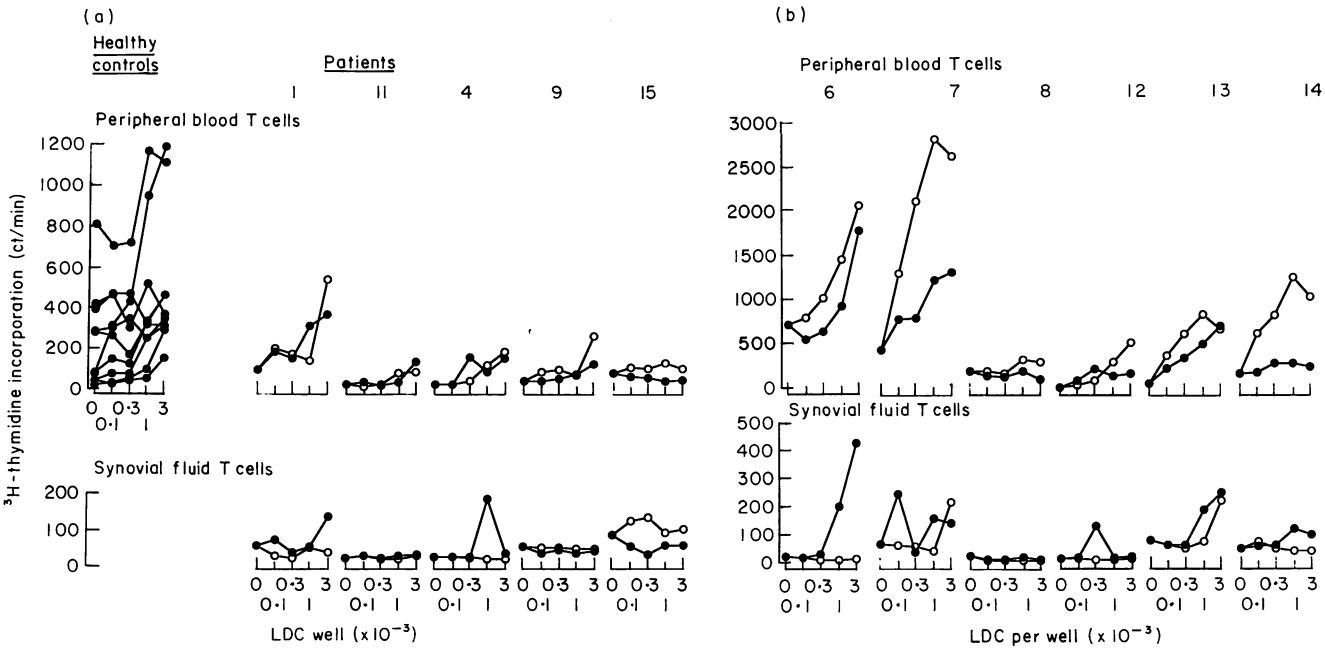

Fig. 4. (a) Stimulation of autologous mixed leucocyte reactions (MLRs) by peripheral blood (PB) (●) and synovial fluid (SF) (○) low density cells (LDC). Patients 1, 11: SARA; patient 4: SARA/ERA; patient 9: RA; patient 15: psoriasis. (b) Stimulation of autologous MLRs by PB (●) and SF (○) LDC from patients 6–8 and 12–14 (USA). For definitions of arthritis types see Materials and methods. Note different scale on graphs of PB and SF T cell responses.

Autologous stimulation of PB lymphocytes

Data from experiments to investigate the ability of PB and SF DC to stimulate autologous MLRs are shown in Fig. 4(a) and (b). LDC isolated from PB of healthy individuals caused a variable degree of proliferation when cultured with autologous T cells (Fig. 4(a). LDC from some individuals caused little autologous stimulation whereas from others there was very marked stimulation.

Stimulation of autologous lymphocytes by paired samples of patient PB and SF LDC is shown in Fig. 4(a) and (b). The data for the USA patients are shown in Fig. 4(b) and for the SARA patients and others in Fig. 4(a). As with healthy controls the level of autologous stimulation of autologous PB lymphocytes was variable. When all patients studied were considered, maximum net levels of stimulation of PB T cells by PB LDC was not different between patients and controls (controls: range 5–1121, mean 341, $n=15$; patients: range 0–1275, mean 361, $n=29$, $P=0{\cdot}83$, not significant). In addition, there were no significant differences between the response of cells from the two major patient groups: those with reactive arthritis and those with undifferentiated monoarthritis. When paired PB and SF LDC were compared in parallel as stimulators they were equally active in seven of 11 patients ($P>0{\cdot}05$) (Fig. 4(a) and (b)). In the remainder, SF LDC were significantly more stimulatory: patient 6 (USA), $P<0{\cdot}05$; patient 7 (USA), $P<0{\cdot}05$; patient 14 (USA), $P<0{\cdot}005$; patient 15 (psoriasis), $P<0{\cdot}025$ (Fig. 4(a) and (b)). In most of these patients PB LDC were also stimulatory, if less so than the SF LDC, but in one USA patient (patient 14) only the SF DC caused convincing stimulation.

Autologous stimulation of SF lymphocytes

Stimulation of autologous SF T cells is also shown for the 11 patients in Fig. 4(a) and (b). Note that the scale of these graphs is different from the PB T cell graphs. Neither PB nor SF LDC caused significant stimulation of autologous SF T cells.

DISCUSSION

In this study we report the isolation of DC-rich low density cells from the synovial fluids of patients with a range of inflammatory arthropathies and show that on a per cell basis they are as potent, or more potent, stimulator cells for MLRs than their counterparts in peripheral blood.

We have also shown that in a minority of patients LDC isolated from SF, but not from PB, caused powerful stimulation of autologous PB lymphocytes. The specificity of this response is unknown but it is suggestive of presentation to reactive lymphocytes of antigen acquired *in vivo* (Knight, 1987a). Harding & Knight (1986) noted that in approximately 10% of children with JCA, SF DC, but not PB DC caused strong stimulation of autologous PB lymphocytes, and similar observations have been made in adults with inflammatory arthritis in studies when unseparated stimulator cells have been used (Crout, McDuffie & Pitts, 1976; Beck *et al.*, 1981; Silver, Redelman & Zvaifler, 1983). However, it should be noted that in the two patients included in the current study with high levels of autologous AMLR stimulated by SF DC, where sufficient cells were available for the comparison to be made, SF DC were also more potent as stimulating allogeneic MLRs (patients 6 and 7, Figs 3 and 4b), suggesting perhaps that they represent the activity of a particularly potent DC rather than presentation of a foreign antigen. Whatever the nature of the stimulus provided by SF DC it appears that T cells isolated from the joint are unable to respond to it, possibly due to chronic stimulation by SF DC *in vivo*.

We and others have reported that synovial fluids from patients with RA (Zvaifler *et al.*, 1985; Harding & Knight, 1986)

and JCA (Tyndall *et al.*, 1983; Harding & Knight, 1986) contain large numbers of DC. In this study the observation was extended to patients with seronegative arthropathies, suggesting that the accumulation of DC is a common feature of joint inflammation and is not specific to a particular disease process. The only known function of DC is to present antigen to T cells and it is tempting to speculate that they are fulfilling this role in arthritic joints and driving the inflammatory reaction. However, direct evidence for this is missing. Despite the accumulation of DC and T cells, these studies showed that T cells within the joint fluid were anergic to syngeneic stimulation by DC. However, in a rat model of arthritis there is evidence that accumulation of antigen-presenting cells precedes the arrival of lymphocytes in the affected joint (Vershure, van Noorden & Dijkstra, 1989).

By electron microscopy peripheral blood LDC comprised monocytes, DC and a few lymphocytes (Knights *et al.*, 1986). We attempted to compare the composition of PB and SF LDC by labelling with the monoclonal antibodies leu M3 (monocytes), UCHT1 (T cells), and an antibody, RFD1, which labels an HLA-D region product with tissue distribution similar to, but distinct from, HLA-DQ (Drexler *et al.*, 1988; Janossy, Campora & Bollum, 1986). This antibody has been reported to label DC but not macrophages in tissue sections (Poulter *et al.*, 1982, 1986) and by immunogold electron microscopy (Knight *et al.*, 1986). Furthermore, removal of $RFD1^+$ cells from LDC abrogates their ability to stimulate MLRs (*vide infra*) strengthening the notion that they are DC. These studies revealed that the composition of PB and SF DC is broadly similar. It should be noted that these preparations contained larger numbers of contaminating lymphocytes than reported in an earlier publication (Knight *et al.*, 1986), probably due to the use of metrizamide of slightly higher density (14·25 compared with 13·7%).

By electron microscopy and immunogold labelling SF DC are larger than their counterparts in PB and express high levels of MHC class II antigen (Knight *et al.*, 1989), an appearance which has been likened to an activation state. Thus, the greater stimulatory activity observed in some MLRs may be related to these qualitative differences. Further work will be required to determine whether these patients represent a clinical subgroup. We have not studied DC isolated from synovial tissue but Waalen *et al.* (1986) have reported that they are more potent than either SF or PB DC as MLR stimulators.

Lymphocytes from human peripheral blood are activated in culture by autologous mononuclear cells (Opeltz *et al.*, 1975). DC are the major stimulators of this response in man (van Voorhis *et al.*, 1982; Kuntz-Crow & Kunkel, 1982) and in mice (Nussenzweig & Steinman, 1980). We found that peripheral blood LDC from healthy controls and arthritis patients caused variable levels of stimulation of autologous peripheral blood lymphocytes with no overall difference between the donor groups. A number of studies have reported that this AMLR response using peripheral blood cells from RA patients is abnormally low (Smith & De Horatius, 1982; Pope *et al.*, 1984). Similar observations have been made in a number of other diseases believed to have an immunological basis, e.g. Sjogren's syndrome (Sauvezie *et al.*, 1982), polymyositis/dermatomyositis (Ranschoff & Distoor, 1983) and myasthenia gravis (Greenberg *et al.*, 1984). However, in none of these studies were enriched DC, the major stimulators of the AMLR, used as stimulator cells. The data presented here demonstrate that patients with inflammatory joint disease may have fewer DC in their peripheral blood than healthy controls, raising the possibility that reduced AMLRs obtained by others with crude cell preparations could reflect reduced numbers of DC within them rather than a 'defect' in responding lymphocytes. Further studies in RA patients with different levels of disease activity, where DC numbers and AMLR responses are studied, may prove informative.

In conclusion, the results illustrate the potent functional activity of SF LDC which are present in large numbers in inflamed joints where they may be presenting antigen, and they emphasize the need to take into account changes in DC numbers when performing studies with crude cell populations from arthritis patients.

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