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Rheumatoid non-lymphoid synovial cells and the induction of mixed leukocyte reactions

Low-density preparations containing Ia$^+$ macrophages and dendritic cells are less stimulatory than peripheral blood non-T cells

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Summary. Interactions of autologous or allogeneic T cells with non-lymphoid synovial cells were investigated to study the mechanisms of T-cell activation in rheumatoid arthritis. The synovial cell fraction obtained by Percoll gradients contained an average of 31% cells positive for monocyte antigens and 62% intensely Ia-positive cells. Tissue cultures demonstrated large numbers of cells with a dendritic morphology. Mixed leukocyte reaction (MLR) cultures between these synovial cell preparations and autologous or allogeneic peripheral blood T cells as responder cells revealed low T-cell responsiveness with an average of 3900 dpm or 17 800 dpm, respectively, in contrast to 16 900 dpm or 79 600 dpm, using rheumatoid peripheral blood non-T cells ($P < 0.01$), despite equivalent amounts of Ia antigens on both stimulator populations, as determined by cell-sorter analysis. The addition of indometacin to these synovial cell/T cell co-cultures resulted in an enhancement of T-cell responsiveness; however, this increase did not reach statistical significance due to large test variations. Co-cultures of non-lymphoid synovial cells and PHA-stimulated autologous T cells induced a marked inhibition of T-cell proliferation that could partially be reversed by the addition of indometacin. The reduction of monocyte-antigen-positive cells by depletion of iron phagocytic cells did not result in a significant enhancement of T-cell responsiveness. These data demonstrate that the majority of non-lymphoid synovial cells, despite the presence of large amounts of Ia-antigens, are not potent inducers of T-cell proliferation and strong suppressing cells in polyclonal T-cell activation. These effects do not appear to reside in the macrophage fraction alone and can only partly be attributed to the action of prostaglandins.

Key words: Rheumatoid arthritis – Autologous mixed leukocyte reaction – Synovial dendritic cells – Synovial macrophages

Introduction

The rheumatoid synovium is characterized by a marked infiltration with T lymphocytes in the presence of activated Ia$^+$ macrophages and dendritic cells and has therefore, many features suggesting an in vivo equivalent to the autologous mixed leukocyte reaction (AMLR). However, this interpretation is complicated by the observations that nearly all synovial T cells are of small, non-blastoid size and although present in large numbers, the Ia$^+$ and/or Tac$^+$ T cells bear only small amounts per cell of these activation markers [1, 2].

Several studies have demonstrated that synovial-fluid mononuclear cells are potent stimulators, both in the autologous and in the allogeneic mixed leukocyte reaction (MLR) and that these cells markedly exceed the stimulatory capacity of corresponding peripheral blood cells [3, 4]. While the exact cell type eliciting this response in the rheumatoid synovial fluid has not yet been identified, the demonstration of cells with a veiled/dendritic morphology in this compartment [5] would suggest that these cells are responsible, especially in view of the observation by Steinman et al. that cells with a similar morphology are the stimulatory cells in peripheral blood MLR [6]. In rheumatoid synovial tissue, large numbers of dendritic cells have been demonstrated by several groups, both in eluted cell preparations [7–10] and in tissue sections [11, 12]. Many of these cells express Ia antigens; they do not bear Fc receptors or monocyte/macrophage markers and are non-phagocytic [7, 13]. While this phenotype along with their dendritic morphology would make them similar to the dendritic cell type described by Steinman et al. [14], several features render these synovial dendritic cells distinct from the classic cell type: (1) the synovial dendritic cells rapidly lose Ia antigen expression upon culture [7]; (2) they are firmly adherent to glass or plastic surfaces, (3) they produce large amounts of collagenase [9]. Especially the last point would suggest that the synovial dendritic cells are related to cells of fibroblastoid origin rather than to those of lymphoid lineage.

In addition to these dendritic synovial cells, which are termed "type II" synovial cells [13], large numbers of intensely Ia$^+$ macrophages designated type I cells are present in the rheumatoid synovium [12, 13]. The present study was undertaken as an initial approach to characterize the interactions between non-lymphoid synovial cell

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preparations containing dendritic cells as well as macrophages and autologous or allogeneic T cells. The question was whether these two synovial cell types can serve as stimulators in MLR cultures probing the hypothesis of an in vivo AMLR in the rheumatoid synovium.

Materials and methods

Patient population. Twenty patients with definite or classic rheumatoid arthritis according to ARA (American Rheumatism Association) criteria and two patients with osteoarthritis without synovitis were studied. The majority of patients ($n=14$) received anti-inflammatory non-steroid drugs and less than 6 mg prednisone; 4 patients were under treatment with either oral ($n=2$) or parenteral ($n=2$) gold salts and 2 patients received D-penicillamine at a dosage of 300 mg/day. Patients receiving more than 6 mg prednisone or cytotoxic drugs were excluded from the study.

Processing of synovial tissue and cell isolation. Synovial tissue obtained at reconstructive surgery was finely minced and incubated with RPMI medium 1640 containing 1 mg/ml collagenase (Sigma, St. Louis, Mo., USA) and 0.15 mg/ml DNAse (Sigma), as previously described in detail [13]. After 60–90 min at 37 °C, the resulting cell suspension was purified upon Ficoll/Isopaque density gradients [13]. Non-lymphoid synovial cells were enriched by means of a discontinuous Percoll (Pharmacia, FRG) density gradient, as reported previously [13]. Cells were harvested from the interface between 20% and 50% Percoll, washed three times, and stored in RPMI 1640 supplemented with 10% fetal calf serum (FCS) or cultured in tissue culture flasks, respectively, to study the cell morphology. This cell population contained less than 5% of cells with lymphoid characteristics as determined by morphology or staining with monoclonal antibodies directed towards T or B lymphocytes. The viability exceeded 95%. Where indicated, phagocytic cells were depleted by carbonyl-iron treatment. Peripheral blood T cells or non-T cells were separated using neuraminidase-treated sheep red blood cells (E) [1].

Identification of cell surface antigens. The following monoclonal antibodies were used for surface antigen identification. The monoclonal antibody 22c6 reacts with a common framework antigen of the Ia molecules [1]. Antibody Mø P-9 (kindly provided by Dr. R. J. Winchester, Hospital for Joint Diseases, New York, NY, USA) detects an antigen present on > 80% of peripheral blood monocytes or tissue macrophages [15]. T-cell antigens were detected by reagents: 89b1 (pan-T cell antibody, 16) and 91d6 (helper/inducer phenotype [16]), both kindly provided by Dr. I. Szer, Hospital for Joint Diseases, NY, USA); OKT8 (suppressor/cytotoxic phenotype [17], Ortho, Raritan, NJ, USA); and anti-Tac (detecting an antigen presumably present on the receptor for interleukin-2, a gift from Dr. T. Waldmann, NIH, Bethesda, USA [18]).

Immunofluorescence and determination of relative antigen density. The aforementioned monoclonal reagents were used in indirect immunofluorescence as previously reported, either using the fluorescence microscope (Leitz, Wetzlar, FRG) or the EPICS V fluorescence-activated cell sorter (Coulter, Hialeah, Fla, USA) [13]. The relative antigen density was determined by plotting the curves for fluorescence intensity in a linear fashion. Subsequently, the control values obtained by staining with an irrelevant monoclonal antibody were subtracted in a channelwise mode. The remaining number of cells in each channel was multiplied by the channel number, arbitrarily beginning with channel no. 50 and ending with no. 256, the last channel displayed. The sum of all channelwise products was calculated and taken as a relative estimation for the amount of antigens of cells applied to the cell sorter. Thus the following formula applied to the calculation:

$$\sum \left[\begin{array}{l}(\text{cell no./channel} + \text{specific antibody} \\ - \text{cell no./channel} + \text{control antibody}) \\ \times \text{channel no. } (50\text{–}256) = \text{relative antigen density}\end{array}\right.$$

All determinations were performed at identical settings of the instrument (gain, laser output and photomultiplier voltage), counting an identical number of cells in all assays. A gain was chosen that did not yield significant numbers of cells in channel 256, thus avoiding a miscalculation of cells that exceeded the maximum fluorescence intensity displayed in the last channel of the instrument. This mode of calculation of the relative Ia antigen density was chosen rather than expressing the results as median channel intensity because only a certain fraction of synovial cells is Ia^+. The median channel intensity, however, would give data regardless of the total number of positive cells, while the calculation used in the present paper takes into account the intensity as well as the number of positive cells.

Mixed leukocyte cultures. RPMI medium 1640 (0.1 ml, supplemented with 10% fetal calf serum and glutamin) containing 1×10^5 E^+ cells was incubated with 0.1 ml irradiated (6000 rad) synovial cells or peripheral blood non-T cells as stimulator cells at cell ratios of 2 : 1 (synovial cells) or 1 : 1 (peripheral blood non-T cells), respectively. Preliminary experiments had indicated that in the assay used in general the maximum response was obtained with these ratios using various responder to stimulator cell ratios varying from 1:0.2 to 1:2 (data not shown). Triplicate cultures were incubated for 7 days, subsequently pulsed with ^{3}H-thymidin, and automatically harvested as previously described [3]. The mean ^{3}H-thymidine uptake of triplicate cultures in dpm (disintegrations per minute) was recorded, and Δ dpm were calculated by subtracting the control values obtained with non-stimulated peripheral blood E^+ cells and non-lymphoid synovial cells or peripheral blood non-T cells, respectively. In parallel experiments, indometacin was added at a concentration of 5 μg/ml which, in preliminary experiments, had been shown to be most effective at this concentration (data not shown). For cell-surface marker analysis at day 7 of culture, cells were cultured at the same ratios in volumes of 2 ml in 24 well-type plates (Nunc, FRG), subsequently harvested, washed, and used for immunoflourescence staining.

Mitogen-induced lymphocyte proliferation. E^+ peripheral blood cells (1×10^5) were stimulated with 1 μg/ml of purified PHA (Wellcome, England, UK) in the absence or presence of 0.5×10^5 autologous non-lymphoid synovial cells. Cells were subsequently cultured in triplicates for a period of 7 days, with or without the addition of 5 μg/ml of indometacin, and harvested as described above. Δdpm were calculated by subtracting the values of non-stimulated control cultures [19].

Statistical analysis. The Mann-Whitney-Wilcoxon test was used to evaluate statistical significance.

Results

Cell-surface analysis and morphological studies

Stimulator cells. The non-lymphoid synovial cell fraction in the layer between 20% and 50% Percoll contained an average of 62% (range 27%–82%) intensely Ia^+ cells, as revealed by monoclonal antibodies 22c6, 31% (range 12%–42%) of monocyte/macrophage antigen positive cells, as shown by reagent Mø P-9, and less than 5% of surface

immunoglobulin-positive cells. The peripheral blood non-T cell fraction obtained by removing E^+ cells contained an average of 59% (range 39%–71%) Ia^+ cells, 45% (range 30%–62%) Mø $P\text{-}9^+$ cells, and 19% (range 12%–32%) surface immunoglobulin-positive cells.

In some instances, parallel cultures of non-lymphoid cells were performed as previously described [13], revealing an average of 35% cells with a morphology typical of rheumatoid synovial dendritic cells [7, 9, 10], while the remainder consisted of cells with a fibroblastoid appearance or the characteristic round spreading morphology of macrophages.

Responder cells. There was no difference with regard to the expression of T-cell surface antigens characteristic of the helper/inducer phenotype ($91d6^+$ cells) or the suppressor/cytotoxic phenotype ($T8^+$ cells) between patients with rheumatoid arthritis and normal controls (data not shown).

The Ia antigen density of non-lymphoid synovial cells markedly exceeds that of autologous peripheral blood non-T cells

Table 1 demonstrates the relative amounts of Ia antigens on six representative non-lymphoid synovial cell preparations in comparison to autologous peripheral blood non-T cells. When equal amounts of cells were analyzed by cytofluorometry, a more than twofold amount of Ia antigens was recorded on the synovial cells. Since in the MLR experiments 50 000 synovial cells were taken as stimulator cells in comparison to 100 000 PB non-T cells, the relative antigen ratio was calculated between half the amounts of Ia antigens present on the synovial cells ($=0.5\times$) and the full amount recorded for the PB non-T cells ($=1\times$). Thus, as shown in the last column of Table 1, roughly equivalent numbers of Ia antigens were present in both stimulator cell populations in the MLR cultures.

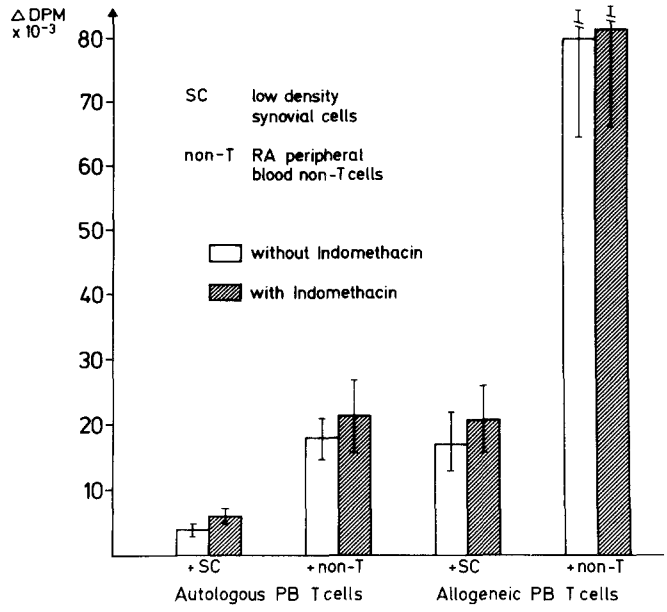

Fig. 1. Comparison of the autologous or allogeneic T-cell response to rheumatoid non-lymphoid synovial cells (SC) or non-T peripheral blood cells ($\Delta dpm \times 10^{-3} \pm SEM$) in the presence or absence of indometacin. In both the autologous or allogeneic situation, PB non-T cells were significantly better stimulators than their synovial counterparts, despite equivalent amounts of Ia antigens (Table 1). The addition of indometacin (5 μg/ml) did not significantly enhance the stimulatory capacity

Less MLR-inducing capacity of non-lymphoid synovial cells in comparison to PB non-T cells

Figure 1 shows the results of all MLR experiments using non-lymphoid synovial cells or PB non-T cells as stimulator cells. These data demonstrate that the synovial cell preparations are significantly less potent stimulator cells in both the autologous and allogeneic cultures when compared to peripheral blood non-T cells. In both situations, the peripheral blood cells were about fivefold more effec-

Table 1. Relative Ia antigen density of non-lymphoid synovial cells markedly exceeds that of autologous peripheral blood non-T cells

Experiment no.	Non-lymphoid synovial cells		Peripheral blood non-T cells	Antigen ratio (SC/2: non-T)
	(1×)	(0.5× = SC/2)	(1×)	
1	16 000[a]	8 000	20 300	0.4
2	34 600	17 300	6 000	2.9
3	72 100	36 100	57 400	0.6
4	81 600	40 800	48 900	0.8
5	139 900	70 000	41 700	1.7
6	152 300	76 200	51 300	1.5
Mean ± SD	82 800 ± 54 800	41 400 ± 27 400	37 600 ± 20 100	1.3 ± 0.9

[a] The relative antigen density was calculated by cell-sorter analysis according to the formula described in Materials and methods

Table 2. Poor MLR stimulatory capacity of non-lymphoid synovial cells as compared to peripheral blood non-T cells. Enhancement by indometacin in some experiments

Expt. no.	Source of T cells	Source of cells added to T cells			
		SC[a]	SC + indometacin[b]	non-T[c]	non-T + indometacin[b]
1	Autologous	1 500[d]	3 000	12 100	ND
2	Autologous	4 300	6 600	20 600	18,400
3	Autologous	1 200	2 100	24 800	35 800
	Allogeneic	3 200	4 900	42 000	31 900
4	Autologous	3 300	5 900	11 600	6 600
	Allogeneic	27 700	47 200	50 100	56 600
5	Autologous	2 900	3 500	7 300	9 500
	Allogeneic	30 500	34 000	86 700	90 500
6	Autologous	1 100	2 000	24 400	35 500
	Allogeneic	42 800	32 000	139 700	144 800

[a] SC non-lymphoid synovial cells obtained by Percoll density gradient centrifugation
[b] Indometacin was added at a concentration of 5 μg/ml
[c] Non-T rheumatoid peripheral blood non-T cells after depletion of E^+ cells
[d] Δdpm = dpm (co-culture) – dpm (T cells non-stimulated) – dpm (non-T/SC)

Table 3. Reduction of iron phagocytic cells does not result in a significant enhancement of T-cell proliferation

Expt. no.	Source of T cells	Source of cells added to T cells			
		SC	SC + indometacin	SC–Fe^+[a]	SC–Fe^+ + indometacin
1	Autologous	15 900	16 300	14 000	16 300
	Allogeneic	34 500	38 400	29 500	37 000
2	Autologous	11 400	12 300	18 000	30 200
	Allogeneic	55 600	48 000	45 100	64 500
3	Allogeneic	43 100	16 800	17 800	29 700

[a] Cells after depletion of Iron phagocytic cells. For further definitions, see Table 2

tive as stimulator cells than their synovial tissue counterparts ($P < 0.01$). The addition of indometacin enhanced the T-cell response by 54% (autologous PB-T + SC), 21% (allogeneic PB-T + SC), or by 19% (autologous PB-T + non-T). However, the increase obtained by indometacin did not restore the stimulating capacity of the synovial cell preparations to a degree comparable to peripheral blood non-T cells and, due to the wide variation of the data obtained, was not statistically significant.

Table 2 shows the intraindividual comparison of the MLR cultures from six representative patients, demonstrating the heterogenous effect of indometacin. In some instances, a marked enhancement induced by indometacin was observed, while in other cultures even an inhibitory effect was seen. The addition of indometacin alone either to peripheral blood or synovial cells did not yield a significant proliferation (data not shown) of otherwise non-stimulated cells.

To investigate the hypothesis that "suppressor macrophages" might inhibit the MLR response, in three experiments phagocytic cells were removed by iron phagocytosis, yielding a synovial cell population containing an average of 5% remaining macrophages, as determined by monoclonal reagent Mø P-9 Table 3 demonstrates that no significant enhancement of the MLR response was observed after the reduction of iron phagocytic cells.

In two patients with non-inflammatory joint diseases, sufficient synovial cells were available to study autologous T-cell responsiveness. In both instances, no significant T-cell proliferation was observed (Δdpm = 0).

Marked reduction of mitogen driven T cell response by the addition of synovial cells

Since apparently prostaglandins were generated during the 7-day MLR cultures, as indicated by the increased responsiveness of T cells after the addition of indometacin, the effect of co-cultures of non-lymphoid synovial cells with PHA-stimulated autologous peripheral blood T cells was tested. Figure 2 demonstrates that the addition of non-lymphoid synovial cells resulted in a marked inhibition of T-cell responsiveness in the presence of this polyclonal T-cell stimulator. When indometacin was added to these

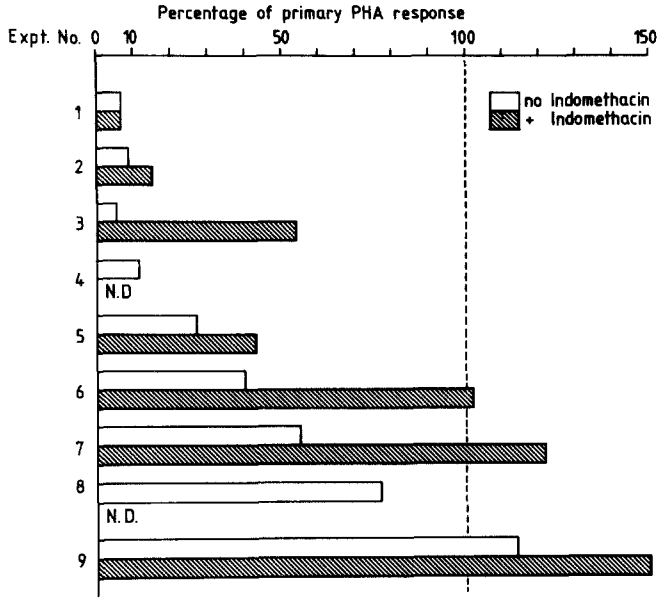

Fig. 2. Influence of the addition of non-lymphoid synovial cells to autologous PHA-stimulated T cells in the presence or absence of indometacin. In the majority of experiments, the addition of synovial cells greatly inhibited mitogenic responsiveness that was partly reversed by indometacin. Results are expressed as percentage of the PHA response in the absence of synovial cells. The addition of indometacin alone did not significantly alter PHA response (data not shown)

cultures, a significant reduction of the inhibitory effect was seen in six of seven experiments. These data indicate that indeed prostaglandins released by non-lymphoid synovial cells were at least in part responsible for the decrease in T-cell responsiveness to PHA.

T-cell phenotype after co-cultures with non-lymphoid synovial cells

T-cell phenotypes were studied after co-culturing with non-lymphoid synovial cells for 7 days in the absence or presence of indometacin. As demonstrated in Table 4, there was no consistent change in T-cell phenotype, indicating that no T-cell subpopulation preferentially responded to the stimulator cells. The number of activated T cells, as demonstrated by the presence of the receptor for interleukin-2 after culture, was low in both the autologous and allogeneic situation (ranging from 2% to 21% of Tac^+ cells).

Table 4. No consistent change of T-cell phenotype after co-cultivation with non-lymphoid synovial cells with or without the addition of indometacin

Experiment no.	Responding T cells	T_{HI}/T_{SC} ratio		
		Day 0	Day 7	
			– indometacin	+ indometacin[a]
1	Autologous	1.8	2.2	2.3
2	Autologous	4.1	2.2	1.0
3	Allogeneic	1.2	1.1	0.8
4	Allogeneic	1.5	1.6	1.6
4	Allogeneic	1.8	1.4	1.0

[a] Indometacin was added at a concentration of 5 μg/ml

Discussion

Two principal findings emerged from the present study: (1) that despite the presence of large amounts of Ia antigens, the majority of non-lymphoid synovial cells are poor stimulator cells in both the autologous and allogeneic MLR and (2) that these cell preparations contain potent suppressing cells in polyclonal T-cell activation, which is due – at least in part – to the action of prostaglandins.

The observation that the synovial cell preparations tested were poor stimulators in the MLR was a quite unexpected one – especially in view of the vast accumulation of activated Ia^+ T cells in the rheumatoid synovium [1] and their close contact to macrophages [20] or interdigitating cells [21, 22] in this compartment. The data obtained complicate the theory of the synovial tissue being an in vivo equivalent of autologous MLR, whereby intensely activated Ia^+ macrophages or other stimulator cells cause a T-cell proliferation at the inflammatory site, thus being the leading event in the subsequent tissue destruction. Evidence was obtained that the poor MLR response observed was not due to "suppressor macrophages" since the reduction of these cells by iron phagocytosis did not result in a significant enhancement of T-cell proliferation. In this respect, the finding that synovial cell preparations from two patients with non-inflammatory joint diseases did not elicit an MLR at all is of special interest, as this tissue is known to contain about 30% Ia^+ macrophages in patients with these diseases [13]. This indicates that synovial macrophages apparently do not act as stimulators in these processes. Furthermore, the poor MLR response appeared not to be due to the action of prostaglandins, since the inhibition of production by indometacin caused only a marginal enhancement of T-cell responsiveness. The poor MLR response of peripheral blood T cells was parallelled by a low number of cells bearing the receptor for interleukin-2, as determined by the analysis of Tac antigen expression. Taken together, these findings argue against the simple hypothesis that in vivo AMLR is caused by the interactions of activated non-lymphoid synovial cells with peripheral blood T cells. In particular, they indicate that the presence of Ia antigens alone on a cell population is not sufficient for a significant MLR response and that other requirements must be fulfilled, presumably the presence of the appropriate stimulator cell type. Similarly, a poor MLR-inducing capacity has been demonstrated using lymphocytes from patients with chronic lymphocytic leukemia as stimulator cells [23], despite the presence of Ia antigens. On the other hand, Ia-negative

B-lymphoblastoid cells are able to activate PHA-stimulated allogeneic T cells further, greatly enhancing the production of IL-2 by normal T cells [24].

Apparently there is a small fraction of potent MLR-stimulator cells in the mixture of synovial cells that fulfill the criteria for the classic dendritic cells described by Steinman, as recently communicated by Førre, as well as Klareskog ([25]; International Symposium on Immunological Aspects of Rheumatoid Arthritis and Related Disorders, Erlangen, FRG, October 1984). However, in view of the results obtained in the present investigation, their number must be rather low and cannot yield a considerable MLR response in a mixture of synovial cells. These data also indicate that the synovial cells with a dendritic morphology, which made up a large proportion of stimulator cells in the present investigation, are distinct from the classic dendritic cells described by Steinman [14].

Thus, the autologous MLR in rheumatoid arthritis is a complicated issue with several conflicting findings that so far have emerged from the studies of this immunoregulatory phenomenon. There is a poor AMLR in peripheral blood [25] and, as shown in the present paper, using a mixture of non-lymphoid synovial cells as stimulator cells. In contrast, a small fraction of synovial cells and autologous synovial fluid lymphocytes [3, 26] are potent AMLR stimulators. These data could be reconciled by the hypothesis that apparently the AMLR is a balanced process that is different in the various compartments and microenvironments present in the inflamed organs. An additional important regulatory mechanism appears to be the action of prostaglandins released by macrophages and possibly cells of fibroblast lineage [10] in the rheumatoid synovium. Their suppressive effect may in part be responsible for the lack of T-cell blasts, the low amount of activation markers per cell on synovial T cells, and the decreased mitogenic responsiveness [1, 2, 19]. Additional experiments are under way to dissect the various immunoregulatory processes that are going on in the rheumatoid synovium, using further defined cell populations.

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