Lymphocytes in rheumatoid and nonrheumatoid synovial fluids

Nonspecificity of high T-cell and low B-cell percentages

L. B. A. van de PUTTE, C. J. L. M. MEIJER, G. J. M. LAFEBER, R. KLEINJAN, and A. CATS

From the Departments of Rheumatology and Pathology of the University Medical Centre, Leiden, The Netherlands

Involvement of the immune system in the pathogenesis of rheumatoid arthritis (RA) is suggested by a variety of immunological features of the disease (Zvaifler, 1973). It is possible that the underlying pathogenetic mechanisms can be elucidated by studying lymphocytes, which play a major role in immune response. The relative proportions of T and B lymphocytes in synovial fluid and peripheral blood have recently been studied in RA (Mellbye and others, 1972; Fröland, Natvig, and Husby, 1973; Vernon-Roberts, Currey, and Perrin, 1974; Sheldon, Papamichail, and Holborow, 1974; Winchester and others, 1974; Brenner, Scheinberg, and Cathcart, 1975; Utsinger, 1975) and also in other joint diseases (Fröland and others, 1973; Brenner and others, 1975; Utsinger, 1975). These studies disagree in several respects, however. An interesting finding in three of these studies was a significant increase in the percentage of T-cells in synovial fluid as compared with that in the peripheral blood of patients with RA (Fröland and others, 1973; Vernon-Roberts and others, 1974; Sheldon and others, 1974). This may represent an expression of cell-mediated immunity in rheumatoid disease, although no data are available for the specificity of this observation for cell-mediated immunity.

The present study was done to find out whether there is a characteristic pattern in the relative proportions of T and B lymphocytes in synovial fluid and peripheral blood in RA. We therefore studied T- and B-cell percentages in paired peripheral blood and synovial fluid samples from patients with RA and other chronic polyarthritides of unknown origin, and also from patients with arthritides generally considered not to be immunologically mediated, including crystal synovitis, traumatic arthritis, osteoarthritis, and pigmented villonodular synovitis.

In addition, absolute numbers of synovial fluid and peripheral blood lymphocytes were determined. In view of the conflicting results of published reports...
we included several controls to find out whether recently recognized sources of potential interference occurring in the investigation of lymphocytes in the rheumatic diseases had influenced the results of our study.

Materials and methods
Peripheral blood and synovial fluids were obtained simultaneously from 27 patients with various forms of arthritis. In addition, peripheral blood from 10 healthy volunteers was studied as a control series.

Patients
The patients were divided into 3 groups according to clinical characteristics and laboratory data. Group I consisted of 12 patients with definite or classical RA as defined by the criteria of Ropes and others (1959). All had positive Waaler-Rose tests and latex fixation tests; none had signs of vasculitis. Group II included 7 patients with other polyarthritides, including 2 cases of psoriatic arthritis, 2 seronegative juvenile RA, 1 adult seronegative HLA-B 27 positive polyarthritis, 1 HLA-B 27 negative sacroiliitis and arthritis, and 1 case with the recently described syndrome of polyarthritis, vasculitis, and hypocomplementaemia (McDuffie and others, 1973). Group III comprised 8 patients with monarthritis, including 3 with crystal synovitis (2 gout, 1 pseudogout), 2 osteoarthrosis, 2 traumatic arthritis, and 1 pigmented villonodular synovitis. Only one of the patients (from group I) received a low dose of prednisone, and none used cytotoxic drugs. Bacterial cultures of all synovial fluids were negative.

Isolation of Mononuclear Cells
Mononuclear cells were isolated from heparinized synovial fluid and peripheral blood samples. Heparinized synovial fluid was first treated with hyaluronidase (BDH Chemicals, Poole, Dorset, 3 mg/10 ml), otherwise the techniques used for cell separation were similar for peripheral blood and synovial fluid. Hyaluronidase did not affect cell membrane properties. After incubation with carbonyl iron (30 mg/10 ml) for 20 minutes at 37°C, phagocytes were removed magnetically. Mononuclear cells were separated from peripheral blood and synovial fluid by density gradient centrifugation according to Boyum (1968). Aliquots of 5 ml were carefully layered on 3 ml Ficoll-Isopaque mixture (density 1077 g/cm³) and centrifuged at 1500 g for 20 minutes. The lymphocyte-rich interface was washed 3 times in RPMI 1640 (Flow Laboratories) and counted in a haemocytometer. Viability as tested by trypan-blue exclusion was always greater than 90%.

Cell Identification
T lymphocytes were detected by spontaneous rosette formation with sheep red blood cells (SRBC) according to Zeylemaker and others (1974). 1 x 10⁶ mononuclear cells were incubated with 50 x 10⁶ SRBC in 1 ml RPMI for 15 minutes at room temperature and centrifuged at 200 g for 5 minutes after which the pellet was incubated at 4°C for at least 90 minutes. After carefully resuspending the rosette suspension, at least 200 mononuclear cells were counted in a haemocytometer. A mononuclear cell with 4 or more adherent SRBC was considered a rosette (E-rosette). The percentage of lymphocytes forming E-rosettes was determined by correcting the values obtained for the proportion of E-rosette-forming mononuclear cells for mononuclear phagocytes, which were determined by nonspecific esterase staining (Yam, Leu, and Crosby, 1971) of mononuclear cells from the interface, using α-naphthyl acetate as a substrate. Only cells showing diffuse cytoplasmic staining were considered to be mononuclear phagocytes. In sedimentation preparations of E-rosettes (Meijer and others, 1976) stained for α-esterase we never found esterase-positive cells in the centre of an E-rosette. Percentages of esterase-positive cells from the interface mononuclear cells tended to be slightly, although not significantly, higher than the percentages of monocytes determined by Giemsa staining. B lymphocytes were identified by the presence of surface membrane immunoglobulins. After the washing procedure 1·10⁴ cells from the lymphocyte-rich interface in RPMI 1640 were incubated at 4°C for 45 minutes with 1 drop of a fluorescein conjugated polyvalent goat-antihuman immunoglobulin antiserum (Nordic, Tilburg, The Netherlands, Lot No. 26–1174) diluted 1:8. The mononuclear cells were washed 3 times in RPMI 1640, resuspended in a drop of buffered glycerol (pH 7.2), mounted under coverslips, and read in an Orthoplan Leitz fluorescence microscope provided with an Osram lamp (HBO 200W) and filters BG 38 and HP 490. The percentage of cells exhibiting surface membrane immunoglobulin fluorescence was determined by comparison with the total number of lymphocyte-like cells estimated after switching each field to phase-contrast light. Cells with the typical macrophase-like appearance were easily recognized by the latter technique and were not included in the count. Percentages of mononuclear phagocytes determined by phase-contrast microscopy correlated well with those obtained by the esterase staining.

An attempt was made to use the complement receptor technique as a second marker for B cells using SRBC coated with a rabbit IgM antibody and fresh mouse complement (Meijer and Lindeman, 1975). However, in the patient groups, but not in the controls, this technique gave an unacceptably wide range of percentages of C3 receptor bearing cells and in several instances virtually zero values in the peripheral blood. These low percentages were found especially in groups I and II. Short-term cultures (see below) gave unequivocal results. Therefore, in this study the C3 receptor was not used as a B-cell marker.

Control Studies
The control experiments were carried out to determine whether cell-free synovial fluid and serum from these patients could have influenced lymphocyte membrane markers under our conditions. In the first of these control experiments T- and B-cells from isolated lymphocyte preparations (20 x 10⁶ cells) from the peripheral blood synovial fluid of 4 RA patients were counted before and after incubation in RPMI 1640 with 10% fetal calf serum for 2 hours at 37°C. In the second experiment isolated peripheral blood lymphocyte preparations (20 x 10⁶ cells) from 3 healthy donors were incubated for 2 hours at 37°C with 1 ml cell-free synovial fluid obtained from 3 patients with
Table I  Mononuclear phagocytes contaminating isolated lymphocyte preparations*

<table>
<thead>
<tr>
<th>Group</th>
<th>PB (%)</th>
<th>SF (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n=12)</td>
<td>5.2</td>
<td>8.2</td>
<td>NS</td>
</tr>
<tr>
<td>Group II (n= 7)</td>
<td>2.0</td>
<td>9.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Group III (n= 8)</td>
<td>6.9</td>
<td>19.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Controls (n=10)</td>
<td>4.0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Percentages of isolated lymphocyte preparations. Mean values calculated by analysis of variance after angular transformation of individual data. PB = peripheral blood; SF = synovial fluid.

RA. In addition, several overnight incubations were carried out as described above for the first and second experiment. Trypan-blue exclusion amounted to more than 90% for the short incubation period and more than 80% for the overnight incubations.

Other Studies

CH₅₀ in serum and synovial fluid was determined according to Kent and Fife (1963) and in synovial fluids was related to protein concentration as determined by the biuret method. Absolute numbers of lymphocytes were calculated by correcting total white cell counts of peripheral blood and synovial fluid for mononuclear phagocytes and other nonlymphoid cells on the basis of esterase staining and Giemsa staining of native peripheral blood and synovial smears respectively. Statistical analysis was performed by an analysis of variance after angular transformation of percentages and after square root transformation of absolute numbers of lymphocytes (Armitage, 1974). P values below 0.05 were considered to be significant.

Results

Mononuclear Phagocytes in Enriched Lymphocyte Preparations

The percentages of mononuclear phagocytes determined by esterase staining of isolated lymphocyte preparations from peripheral blood and synovial fluid are given in Table I. In all 3 groups contamination by esterase-positive cells was greater in synovial fluid lymphocyte preparations than in peripheral blood lymphocyte preparations.

Lymphocyte Studies

The percentages of both T and B lymphocytes in synovial fluid and peripheral blood were significantly different, as shown in Table II. Percentages of T lymphocytes were always higher in synovial fluids than in peripheral blood, whereas percentages of B lymphocytes in synovial fluids were consistently very low and occasionally nil. There was no significant difference in peripheral blood T or B lymphocyte percentages between any of the patient groups and the control group. Absolute numbers of synovial fluid lymphocytes were significantly higher in groups I and II as compared with group III (Table III), whereas absolute numbers of peripheral blood lymphocytes were significantly lower in groups I and II than in the control group.

Control Studies

Short-term culture of isolated lymphocyte preparations made from the peripheral blood and synovial fluid of 4 patients with RA did not significantly alter the percentages of either T- or B-cells. Percentages of synovial fluid B-cells even tended to be lower at the end of the 2-hour culture period. Isolated lymphocyte preparations from 3 healthy donors were incubated with cell-free rheumatoid synovial fluids for 2 hours to exclude the possibility that some constituent of the synovial fluid might have influenced the lymphocytes in such a way as to result in increased T-cell and decreased B-cell percentages. No such influence could be shown and no significant differences were found between T- and B-cell percentages before and after culture. Several overnight cultures gave the same result.

Table II  Percentages of T and B lymphocytes in peripheral blood and synovial fluid*

<table>
<thead>
<tr>
<th>Group</th>
<th>PB (%)</th>
<th>SF (%)</th>
<th>T-cells</th>
<th>B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n=12)</td>
<td>66.4</td>
<td>82.4</td>
<td>&lt;0.001</td>
<td>10.5</td>
</tr>
<tr>
<td>Group II (n= 7)</td>
<td>65.8</td>
<td>80.0</td>
<td>&lt;0.005</td>
<td>9.2</td>
</tr>
<tr>
<td>Group III (n= 8)</td>
<td>63.5</td>
<td>83.5</td>
<td>&lt;0.001</td>
<td>9.7</td>
</tr>
<tr>
<td>Controls (n=10)</td>
<td>62.2</td>
<td>—</td>
<td>—</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* Percentages of total lymphocytes. Mean values calculated by analysis of variance after angular transformation of individual data.

Table III  Absolute numbers of synovial fluid and peripheral blood lymphocytes/mm³*

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td>1311</td>
<td>1353</td>
<td>1535</td>
<td>1729</td>
</tr>
<tr>
<td>SF</td>
<td>2131</td>
<td>1357</td>
<td>509</td>
<td>—</td>
</tr>
</tbody>
</table>

* Mean values calculated by an analysis of variance after square root transformation of individual data.
OTHER STUDIES

No significant correlation was found between the absolute number of synovial fluid lymphocytes and parameters for the degree of joint inflammation such as total white cell count, granulocyte count, or CH50 activity in the synovial fluid.

Discussion

Our results show that the percentages of T and B lymphocytes in synovial fluid differed significantly from those in the paired peripheral blood samples. The remarkably constant relative proportions of T and B cells in synovial fluids were shown in a wide variety of arthritides, including RA and other chronic polyarthritis, as well as in arthritides generally considered not to be immunologically mediated, e.g. crystal synovitis and traumatic arthritis. Therefore, the characteristic pattern of high T-cell and very low B-cell percentages in synovial fluids seems to be a general feature of inflammatory exudates and cannot be considered an expression of cell-mediated immunity in itself. On the other hand, the absolute numbers of (predominantly T) lymphocytes were significantly increased in synovial fluids from patients with RA and other polyarthritis of unknown origin, and this may indeed be related to cell-mediated immunity in these diseases which is suggested by the recent demonstration of T-cell predominance in rheumatoid synovial membranes (Boxel and Paget, 1975; Meijer and others, 1976) and the presence of lymphokines in rheumatoid synovial fluids (Stassny and others, 1973). In the peripheral blood the absolute number of lymphocytes was decreased in groups I and II (RA and other polyarthritis) compared with the control values. This finding agrees with a recent report by Utsinger (1975). We were unable to show a correlation between absolute numbers of T lymphocytes and complement activity in the synovial fluid as reported by others (Winchester and others, 1973).

Recent studies on T- and B-cells in RA, especially those concerned with synovial fluid lymphocytes, have shown considerable discrepancies. Our data agree with those of Freland and others (1973) and Sheldon and others (1974) as far as the proportional pattern of T- and B-cells in rheumatoid synovial fluids is concerned, even though the percentages differ considerably. However, other authors have reported different results, i.e. decreased (Winchester and others, 1974) or equal (Utsinger, 1975) percentages of T-cells and increased (Mellbye and others, 1972) or equal (Vernon-Roberts and others, 1974; Brenner and others, 1975; Utsinger, 1975) percentages of B-cells in synovial fluids as compared with peripheral blood. Very few data on lymphocytes in nonrheumatoid synovial fluids are available.

Brenner and others (1975), who investigated synovial fluid T and B lymphocytes in diseases comparable with our group II, found different proportions of these cells in their individual patient groups. On the other hand, Utsinger (1975) recently studied a wide variety of arthritides including RA, osteoarthritis, and gout, and was unable to show any difference between the percentages of T- and B-cells in the synovial fluids, and also found the same pattern of T- and B-cell percentages in the peripheral blood and synovial fluids of all of the groups he investigated.

The discrepancies in the studies mentioned above probably partly reflect differences in the methods applied. In this respect several sources of potential interference with the investigation of lymphocytes in these diseases may be relevant, i.e. the greater contamination of isolated synovial fluid lymphocyte preparations by mononuclear phagocytes, as shown by our data; the presence of antilymphocyte antibodies (Winchester and others, 1974) in the peripheral blood; and probably the presence of lymphokines in some synovial fluids as shown by Stassny and others (1973). In our patient groups the percentages of mononuclear phagocytes, as shown by the esterase staining, were higher in isolated synovial fluid lymphocyte preparations than in those from peripheral blood. Thus omission of the correction of the T-cell percentages for mononuclear phagocytes would have resulted in a less significant difference between synovial fluid and peripheral blood in this respect.

The presence of antilymphocyte antibodies might result in positive membrane immunofluorescence and thus lead to a false increase of B lymphocyte percentages (Winchester and others, 1974). Short culturing of lymphocytes has been reported to correct these phenomena (Messner, Kennedy, and Jelinek, 1975). In our short incubation experiments T- and B-cell percentages of both peripheral blood and synovial fluid from RA patients did not change. In addition, we found no significant differences in the percentages of peripheral blood T- and B-cells between patient groups and controls, which suggests that antilymphocyte antibodies had not interfered to any significant degree with the accurate determination of lymphocyte surface markers by our methods. In addition to antibodies and complexes synovial fluid might also contain many other substances capable of influencing lymphocyte membrane properties. It was interesting that incubation of normal lymphocytes in synovial fluids did not alter membrane markers in such a way as to result in an increase of E-rosette forming cells or a decrease of membrane Ig-positive cells as shown by both short-term and overnight culture studies. It therefore seems unlikely that with the methods used in our studies environmental influences interfered to any
significant degree at least with regard to the accurate determination of E-rosetting and detection of membrane Ig, which are generally accepted as specific markers for T- and B-cells, respectively (World Health Organization, 1974).

The meaning of the relative increase of T-cells and the relative decrease of B-cells in synovial fluids remains speculative for the time being. Recently, Manconi and others (1976) found a similar pattern of T- and B-cell percentages in another extravascular fluid, i.e. cerebrospinal fluid. One possibility, therefore, is that this phenomenon reflects only functional qualities of these cells, the predominance of T-cells being an expression of their recirculation pattern.

We are grateful to Dr. H. Mattie for statistical evaluation of the results; to Mrs. Henny J. P. Belt for typing the manuscript; and to Mrs. Carla van der Star for technical assistance.

References


BRENNER, A. L., SCHEINBERG, M. A., AND CATHCART, E. S. (1975) Arthr. and Rheum., 18, 297 (Surface characteristics of synovial-fluid and peripheral-blood lymphocytes in inflammatory arthritis)


SHELDON, P. J., PAPAMICHAIL, M., AND HOLBOROW, E. J. (1974) Ibid., 33, 509 (Studies on synovial fluid lymphocytes in rheumatoid arthritis)


UTSINGER, P. D. (1975) Ibid., 18, 595 (Synovial-fluid lymphocytes in rheumatoid arthritis)


