Studies on synovial fluid lymphocytes in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic disease in which the major lesion is a chronic inflammation of the synovial membrane, with a characteristic though not pathognomonic histology. Rheumatoid activity in the synovial membrane may lead to the production of an increased amount of synovial fluid which in active cases is often richly cellular with polymorphs, monocyte-like cells, or lymphocytes predominating. The aetiology of the condition is unknown but the presence in many cases of serum rheumatoid factor, and often of increased circulating immunoglobulin and other auto-antibodies, has for long suggested involvement of the immune system. Lymphocytes are known to be closely involved in immune responses leading to graft or tumour rejection, antibody production, and delayed hypersensitivity, and it seemed prudent therefore to study their characteristics in the peripheral blood of controls and RA cases and in synovial effusions occurring in patients with RA, seropositive or negative.

Lymphocytes may be identified as thymic-derived (T) or bone marrow-derived (B) cells. Both are involved as effectors of immune responses, though the former are especially concerned with the expression of cell mediated immunity, i.e. delayed hypersensitivity, and the latter as the precursors of plasma cells producing circulating antibodies. T cells are also important as helper cells in the response to antigens—though co-operation with other cells in all these immune processes is likely.

Human T cells do not differ morphologically from B cells by ordinary light microscopy. Electron microscopy reveals differences in surface conformation (Polliack, Lampen, Clarkson, De Harven, Bentwich, Siegal, and Kunkel, 1973). T cells may be detected by their ability to form rosettes with sheep red cells (Lay, Mendes, Bianco, and Nussenzweig, 1971; Jondal, Holm, and Wigzell, 1972; Papamichail, Holborow, Keith, and Currey, 1972). Additionally, they respond to the mitogen phytohaemagglutinin (PHA) more readily than B cells (Greaves, Owen, and Raff, 1973). B cells may be distinguished from T cells by the presence of surface immunoglobulins (Papamichail, Brown, and Hol-

borow, 1971; Frøland and Natvig, 1971) readily demonstrable as membrane fluorescence when lymphocytes are treated with antihuman immunoglobulin conjugated with fluorescein.

This study was carried out in order to enumerate the T and B cells in blood and synovial fluid in cases of RA, and to test the functional capacity of the lymphocytes.

Materials and methods

Forty patients with RA were investigated. In 12 cases peripheral blood was examined. In 20 cases synovial fluid and in 8 cases paired samples of blood and synovial fluid were studied. There were 20 control blood samples obtained from laboratory staff and hospital inpatients, awaiting minor surgery for non-rheumatoid conditions.

LYMPHOCYTE SEPARATION

(a) Peripheral blood

20 ml venous blood was defibrinated and the red blood cells were sedimented by mixing with Plasmagel (Laboratoire Roger Bellon) and incubating at 37°C for 5–10 mins. The buffy coat was then layered onto 12 ml of Ficoll-metrizoate mixture (Lymphoprep, Nyegaard, Oslo) and centrifuged at 1,400 g for 25 mins. The cells at the interface were recovered and washed twice in medium 199.

(b) Synovial fluid

Synovial fluid was obtained by needle aspiration and mixed with 1,250 units heparin and 1,500 units hyalase per 20 ml fluid. The fluid was incubated at 37°C for 15 mins while rotating in a Matburn mixer. The fluid was then filtered through sterile gauze, and centrifuged at 200 g for 5 mins. The cell pellet was resuspended in medium 199 and layered onto Lymphoprep, centrifuged at 1,400 g for 25 mins, and the interface cells removed and washed twice in medium 199.

SURFACE IMMUNOGLOBULIN STAINING (B CELLS)

$1-2 \times 10^6$ cells were mixed with five drops 1/5 fluoresceinated sheep antihuman immunoglobulin serum (Wellcome Reagents) and incubated at 4°C for half an hour. They were washed twice in medium 199, resuspended in 50% buffered glycerol (pH 8.0), and examined by epillumination with a Zeiss binocular photomicroscope equipped as previously described (Papamichail and others, 1972). The percentage of cells exhibiting surface immunofluorescence was enumerated by comparison with the

total number estimated on switching each field to transmitted light.

SPONTANEOUS ROSETTES (T CELLS)

A 0.5% suspension of washed sheep red blood cells was prepared. 0.5 ml was added to 2×10^6 lymphocytes in 0.6 ml medium 199 in a 7.5×1 cm plastic round-bottomed tube. After mixing the cells were kept at room temperature for 10 mins, then centrifuged for 5 mins at 300 *g* and incubated at 4°C for 60 mins. The cells were then gently resuspended by slow rotation of the tubes for about 30 secs on a Matburn mixer. One drop of the resultant suspension was mounted under a coverslip and examined for rosettes with a binocular microscope ($\times 6$ eyepieces, $\times 10$ objective). A rosette was defined as a white blood cell with at least 4 sheep red blood cells attached. At least 200 white cells were counted. For both tests the variation in counts by one observer, or between observers, was $\pm 5\%$.

LYMPHOCYTE TRANSFORMATION BY PHYTOHEMAGGLUTININ (PHA)

1×10^6 lymphocytes in 1 ml RPMI 1640 (Bio-Cult Laboratories) supplemented with 10% fetal calf serum, Hepes buffer 1M (Bio-Cult Laboratories), L-glutamine, and antibiotics were cultured in triplicate in 2.5 ml plastic culture tubes (Stayne Laboratories). PHA (Wellcome Reagents) 10 μ l was added to each culture tube and incubated at 37°C for 72 hrs. 20 μ l 14 C-thymidine (0.08 μ Ci) was added to the culture 6 hrs before harvesting. The cells were washed twice and resuspended in 0.3 ml saline solution. 0.1 ml of the suspension was placed on a filter disc (Whatman 3MM) and dried. The discs were processed sequentially in 10% trichloroacetic acid, 3% perchloric acid, ethanol, and ether, and transferred into scintillation fluid; radioactivity was measured in a liquid scintillation counter.

CYTOCENTRIFUGE PREPARATIONS

Three drops of the rosette suspension were diluted with twelve drops medium 199. Four drops of the resultant suspension were cytocentrifuged and the preparation fixed and stained with Giemsa. Preparations containing less than 80% lymphocytes were rejected.

Results

The percentage of T cells was seen to be somewhat higher in rheumatoid synovial fluid (72.5) than in rheumatoid peripheral blood (66.4) or the blood of control subjects (66.9), though the difference does not reach statistical significance. Whereas the percentages of B cells in the peripheral blood of controls (17.1) and patients with RA (19.2) were very similar, there was a significant reduction in the percentage of B cells in synovial fluid (5.6, $P < 0.001$) (Fig. 1).

In 8 cases where matched blood and synovial fluid lymphocytes were compared (Table I), the results can be seen to be similar to those found in the unmatched group, though the increase in the proportion of T cells as well as the decrease in proportion of B cells was statistically significant ($P = 0.025$ for T cells; $P < 0.05$ for B cells).

FIG. 1 Percentages of T and B cells in the blood and synovial fluid of patients with RA, and in the blood of control subjects

Table I T and B cell percentages in paired blood and synovial fluid (8 patients)

T cells		B cells	
Blood	Synovial fluid	Blood	Synovial fluid
80	84	9	5.5
60	74	28	23
67	74	33	5.5
72	88	26	5
70	76	9	7
70	86	ND	8
61	60	10	9
70	87	12.5	0.5
Mean	68.8	18.2	7.9
	$P = 0.025$		$P < 0.05$

Morphologically the rosettes formed from synovial fluid lymphocytes exhibited a greater tendency than those formed from peripheral blood (Fig. 2a) to join together forming multiple rosettes (Fig. 2b). Also, many had a larger number of sheep erythrocytes attached.

The results of PHA stimulation can be seen in

FIG. 2 Cytocentrifuge preparations of Giemsa-stained rosettes from (a) peripheral blood and (b) synovial fluid of an RA patient, showing formation of multiple rosettes

FIG. 3 The PHA responses of lymphocytes from peripheral blood and synovial fluid of RA patients and from the blood of control subjects

Table II PHA responses in paired blood and synovial fluids (uptake ^{14}C thymidine cpm)

Blood	Synovial fluid
34,164	5,091
11,203	2,370
10,974	5,097
18,148	788
13,246	7,550
15,034	1,242
13,530	195
15,084	1,282
Mean 16,429	2,952

$P < 0.001$

Fig. 3. There was marked lack of response by the synovial fluid lymphocytes (mean, 5,079 cpm) compared with those from rheumatoid (mean, 15,490 cpm) or control blood (mean, 16,898 cpm) ($P < 0.001$). The results comparing paired blood and synovial fluid are shown in Table II, from which again the degree of PHA unresponsiveness of synovial fluid lymphocytes will be apparent.

Discussion

An increase in the T cell and diminution in the B cell proportions in rheumatoid synovial fluid compared to rheumatoid peripheral blood has been

previously reported (Frøland, Natvig, and Husby, 1973; Winchester, Siegal, Bentwich, and Kunkel, 1973). Our results are in agreement with these. We did not find an increase in the percentage of surface immunoglobulin bearing cells in rheumatoid synovial fluid compared to peripheral blood of the same patient as has been reported (Mellbye, Messner, Debord, and Williams, 1972). The paucity of B cells in synovial fluid may occur because they are rapidly differentiating into plasma cells which are commonly seen in considerable numbers in inflamed synovial membranes. Another possible explanation is that B lymphocytes escape out into synovial fluid less easily than T lymphocytes. The increased percentage of T

cells in fluid may occur as a result of their proliferation *in vivo*, as a result of antigenic stimulation.

It has been previously reported that synovial fluid lymphocytes seem to respond poorly to PHA (Panayi, 1973) and our study confirms this.

PHA unresponsiveness may arise as a result of failure of PHA to attach to the cell on account of blocking of the receptor sites. This could occur as a result of competition by some synovial fluid constituent for binding sites for PHA. However, the addition of fluorescein-conjugated PHA to synovial fluid lymphocytes showed no evidence of lack of staining. Therefore, we do not feel that blocking of receptor sites has occurred, but that there is a greater likelihood that the cells have already been transformed *in vivo*. This being the case, the cells would be unable to respond further to the mitogenic effect of PHA. In favour of this hypothesis is the observation that spontaneous uptake of isotopically-labelled thymidine by synovial fluid lymphocytes was in most cases greater than for peripheral blood lymphocytes, in a separate series of experiments. Also lymphocyte transformation of autologous lymphocytes by rheumatoid synovial fluid, but not by non-rheumatoid synovial fluid, has been shown (Kinsella, 1973).

Viral infection is known to suppress the response of lymphocytes to PHA. This has been reported in rubella (Olson, South, and Good, 1967), hepatitis (Willems, Melnick, and Rawls, 1969), and infectious mononucleosis (Rubin, 1966; Sheldon, Papamichail, Hemsted, and Holborow, 1973). In the latter condition also we have found that the peripheral blood lymphocytes, including the atypical mononuclear cells characteristic of the condition, form multiple rosettes similar to those described above.

None of the patients in the study were receiving cytotoxic drugs, although in the dosages usually employed in the treatment of RA it has been shown that they do not significantly interfere with immune responsiveness (Denman, Denman, Greenwood, Gall, and Heath, 1970). Chloroquine on the other hand may do so (Panayi, Neill, Duthie, and McCormick, 1973), but none of the patients had received this drug.

The increased proportion of T lymphocytes in synovial fluid in RA suggests that their role in the pathogenesis of the inflammatory process should be further investigated. Evidence for cell mediated responsiveness to aggregated IgG in RA has been reported (Frøland and Gaarder, 1971; Brostoff, Howell, and Roitt, 1973). Lymphokines released by antigen-stimulated T cells may contribute to the inflammatory process and have been shown in rheumatoid synovial fluid (Ziff, 1973). The aggregation of rosettes formed by synovial fluid lymphocytes may be due to the release of lymphokine(s) with cell aggregating properties by activated T cells. B cells may be of lesser importance in chronic arthritis

since it has been shown that in chickens, neonatal bursectomy, followed by the injection of intra-articular antigen (Oates, Maini, Payne, and Dumonde, 1971), and in humans, infantile X-linked agammaglobulinaemia (Bruton type)—both characterized by B cell deficiency—may be associated with a chronic inflammatory arthritis. Additionally, proliferating T cells in inflamed joints may have an enhancing effect on local antibody production with resulting formation of immune complexes (Ziff, 1973).

Further study of T and B cell function in RA and the effects of manipulation of cell populations, e.g. by immunosuppressive therapy or by immune stimulating factors may help clarify the immune mechanisms involved and lead to more effective treatment.

Summary

Lymphocytes were studied in forty cases of RA. 20 synovial fluids and 12 peripheral blood samples were obtained. A further 8 paired samples of synovial fluid and blood and 20 control blood samples were also examined.

The overall mean percentages of T cells in synovial fluid and rheumatoid peripheral blood were 72.5 and 66.4, respectively, and in the paired synovial fluid and blood samples 78.6 and 68.8 ($P = 0.025$). The mean percentages of B cells were 5.6 (synovial fluid) and 19.2 (peripheral blood) ($P < 0.001$). There was no significant difference between the T and B cell percentages in rheumatoid and control peripheral blood.

The spontaneous rosettes formed by synovial fluid lymphocytes with washed sheep erythrocytes showed a greater tendency to join together than did the rosettes formed by peripheral blood lymphocytes.

The results of PHA stimulation showed marked unresponsiveness of synovial fluid lymphocytes whereas the lymphocytes in peripheral blood responded similarly to controls.

It is concluded that there is a relative increase in the ratio of T to B lymphocytes in rheumatoid synovial fluid compared to peripheral blood and that the lymphocytes are relatively resistant to stimulation by PHA possibly due to prior stimulation *in vivo*.

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DISCUSSION

DR. KENNEDY (*Glasgow*) Do you have any information as to the time of onset of the effusion, or as to when you took off the samples of synovial fluid? Did you do serial samples to determine whether the proportions of T cells to B cells altered?

DR. SHELDON We didn't carry out serial studies. The duration of disease and the duration of the effusion did vary; some cases were acute and some had recurring effusions going back over several months.

DR. J. M. H. MOLL (*Sheffield*) Did you separate your seropositive from your seronegative results?

DR. SHELDON There did not seem to be any obvious difference.

DR. B. VERNON-ROBERTS (*London*) Dr. Currey and I have just completed a study of 30 cases where we have taken paired samples of rheumatoid synovial fluid and blood (Vernon-Roberts, Currey, and Perrin, 1974). We found a significantly higher proportion of cells bearing surface IgA in synovial fluid. Did you separate B cells into the various subclasses?

DR. SHELDON In several instances we did apply the appropriate antisera for the subgroups but we did not find any cases of increased IgA. In fact this would perhaps be difficult to substantiate because the percentage of synovial cells exhibiting immunofluorescence was often so small that to find an increased percentage of IgA would be rather difficult.

DR. B. VERNON-ROBERTS (*London*) Well, in our studies the mean levels of B cells in both blood and synovial fluid were about 45% and for T cells we had a mean of 38% in blood and 49% in synovial fluid. In other words, we agree there is a higher level of T cells in synovial fluid. We found no evidence of a higher level of B cells.

DR. SHELDON Did you verify the purity of your lymphocyte population in all these cases?

DR. B. VERNON-ROBERTS (*London*) We did.

DR. SHELDON We have found that when one has rather impure cell populations with 20 or 40% macrophages, these commonly show surface staining when one adds anti whole immunoglobulin but we have not tried anti IgA.

DR. R. MAINI (*Kennedy Institute*) We should draw the attention of the audience to the study (Mellbye and others, 1972) which showed a different result from yours in that they reported an increased percentage of B cells in the synovial fluid. I am glad there are an increasing number of laboratories now agreeing about the findings you reported today. I also recall the findings of various workers drawing attention to the fact that there are a large number of lymphocytes in the synovial exudates of rheumatoid patients which do not label with either T or B cell markers—these have been termed 'nul' cells. Would you tell us whether your T cells and B cell populations in blood and synovial fluid added up to 100% and whether you observed any of these 'nul' cells.

DR. SHELDON I would say that we usually fail when adding T and B cells to reach 100% and this could be due to 'nul' cells. On the other hand, there is often difficulty in obtaining a really pure population of lymphocytes. The 'nul' cells may be macrophages or they may be a small subpopulation of lymphocytes, or a combination of both.

DR. R. MAINI (*Kennedy Institute*) Your finding of poor PHA responses of synovial fluid cells might be explained if Mellbye's results of an increase in B cells were correct. Your alternative explanation that the lymphocytes are already transformed should be easily confirmed by an examination of the cells—did you look at these?

DR. SHELDON Many of the cells separated from synovial fluid have the appearance of transformed cells. If they are B cells as Mellbye pointed out then I don't think we would have obtained such high percentages of rosette-forming cells since B cells don't form rosettes with masked uncoated sheep cells and neither do macrophages.

DR. P. J. L. HOLT (*Manchester*) There is a big artifact in all this sort of work. When you sediment lymphocytes through a gradient the proportion of B and T cells which you get out can bear a very varying relationship to the cells that you started off with. When done in replica one sometimes gets quite different results. This could seriously affect your results.

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