T and B cells in the blood and synovial fluid of rheumatoid patients

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The simultaneous application of the techniques of immunofluorescent staining for the presence of surface membrane immunoglobulin and spontaneous rosette formation with sheep erythrocytes allows the identification of subpopulations of human peripheral blood lymphocytes corresponding with bone marrow-derived (B) cells and thymus-derived (T) cells identified in other ways (Papamichail, Holborow, Keith, and Currey, 1972). These techniques have been applied to the study of cells in rheumatoid synovial effusions and to lymphocytes from peripheral blood obtained on the same occasion.

Materials and methods

SEPARATION OF LYMPHOCYTES

Samples of heparinized venous blood and heparinized synovial fluid from knee joint effusions were obtained on the same occasion from each of 30 patients with rheumatoid arthritis. None of the patients was receiving corticosteroid or immunosuppressive therapy. Total and differential leucocyte counts were performed. The removal of erythrocytes from the blood samples was achieved by mixing 4 parts of blood with 1 of Plasmagel (Roger Bellon, Neuilly) and allowing the erythrocytes to sediment at 37°C for 30 minutes. 10–15 ml of the leucocyte-rich supernatant from the Plasmagel-treated blood and the leucocyte-rich lightly-centrifuged synovial fluid (not treated with Plasmagel) were then layered carefully onto 10 ml of a mixture of 6% Ficoll (Pharmacia, Sweden) and 13.3% triosyl '75' (Glaxo Laboratories, Greenford, England) and centrifuged at 2000 *g* for 20 minutes at room temperature. The resultant layer above the interface was rich in lymphocyte-like cells, and this layer was aspirated and washed 3 times in medium 199. The cells obtained by this method from either blood or synovial fluid were examined by phase-contrast microscopy of unstained wet preparations and conventional microscopy of stained smears; they consisted of 95% lymphocyte-like cells and 5% monocyte-like cells with occasional polymorphs.

ROSETTE FORMATION

For rosette formation about 3×10^6 lymphocytes in 0.4 ml of medium 199 were mixed in a tube with an equal volume of a 0.5% suspension of washed sheep red blood cells (SRBC) in Hanks's solution (pH 7.2) containing

10% fetal calf serum absorbed with SRBC. After mixing, the cell mixture was centrifuged at 150–200 *g* for 5 minutes at room temperature, and then kept at 4°C for 30 minutes. The pellet of cells was gently resuspended by rotating the tube on a Matburn mixer at 28 r.p.m. Samples were then withdrawn and the percentage of lymphocytes forming rosettes (with a minimum of 5 attached SRBC) was counted in a haemocytometer chamber using phase-contrast. With some samples membrane immunofluorescence and rosetting with SRBC were combined by first exposing the lymphocytes to conjugates diluted 1/5–1/10 and then washing twice before mixing with SRBC as above.

MEMBRANE IMMUNOFLUORESCENT STAINING

The following fluorescein-conjugated antisera were used at 1/5 or 1/10 dilution: sheep antihuman immunoglobulin, sheep antihuman IgG, sheep antihuman IgM, sheep antihuman IgA (Wellcome Research Laboratories, Beckenham, England). All the conjugates were deaggregated by ultracentrifugation before use, and their specificity was checked by standard methods (Papamichail, Brown, and Holborow, 1971). Staining was carried out by suspending 3×10^6 washed lymphocytes in 0.1 ml of the appropriate diluted conjugate and incubating at 37°C for 30 minutes. The conjugate was then removed and the cells were washed 3 times in Hanks's solution (pH 7.2) and were mounted under coverslips in the same medium. Membrane immunofluorescence was looked for by epi-illumination with a Zeiss binocular photomicroscope (HBO-200 mercury vapour light source, BG 38 and BG 3 exciter filters, 460 nm reflector, 100 × oil neofluar objective, 10 × wide-angle eye-pieces, barrier filter with cutoff below 500 nm). The morphology of cells exhibiting membrane immunofluorescence and cells or rosettes not exhibiting membrane immunofluorescence within each field was examined by changing from epi-illumination with ultra-violet light to phase-contrast examination of each cell by transmitted tungsten illumination. At least 500 cells were examined in this way in each preparation.

IMMUNOGLOBULIN LEVELS IN BLOOD AND SYNOVIAL FLUID

Samples of blood and synovial fluid were stored at –20°C until tested. The method of radial immunodiffusion (Mancini, Carbonara, and Heremans, 1965) was used to determine the levels of immunoglobulins A, G, and M using Tri-Partigen immunodiffusion plates (Hoechst Pharmaceuticals).

Results

ROSETTE-FORMING CELLS

The mean percentage of rosette-forming cells (RFC) in the synovial fluid (49%) was significantly higher ($P < 0.01$) than in the peripheral blood (38%) of the same group of patients (Table I), though the range was of the same order of magnitude (10–70%) in both cases (Table II). The percentage of lymphocytes forming rosettes was of the same order of magnitude in the blood and synovial fluid of one-third of the patients. The increase in the mean percentage of RFC in synovial fluid relative to the blood was not accompanied by a concomitant fall in the mean percentage of B cells in synovial fluid (which was the same as that of the blood): however, the differences in the percentage of RFC in synovial fluid and blood were reflected by similar differences in the percentage of 'null cells' (Table I) which were morphologically indistinguishable from small lymphocytes but did not form rosettes or possess surface immunoglobulin.

Table I Percentages of lymphocyte types in blood and synovial fluid of 30 rheumatoid patients

Lymphocyte types	Mean percentage cells		Significance of difference of means
	Blood	Synovial fluid	
B	44	43	$P > 0.05$
T	38	49	$P < 0.01$
B + T	82	92	$P < 0.001$
'Null cells' (100 - B + T)	18	8	$P < 0.001$

IMMUNOGLOBULIN-BEARING LYMPHOCYTES

After staining with polyvalent antihuman Ig there was no significant difference between the mean percentage of lymphocytes showing surface staining for Ig between synovial fluid (43%) and blood (44%), though a wide range of values was observed in both cases (Tables I, II, and III). In support of these findings there was also no significant difference between the mean combined total percentage of

Table II Percentage of B-cells and T-cells in the blood and synovial fluid in each of 30 rheumatoid patients

Case no.	Percentage cells					
	Blood			Synovial fluid		
	B	T	B + T	B	T	B + T
1	34	29	63	42	56	98
2	64	26	90	57	31	88
3	38	58	96	32	64	96
4	31	30	61	52	68	120
5	39	33	72	20	66	86
6	65	15	80	41	57	98
7	74	22	96	42	51	93
8	41	49	90	40	63	103
9	36	44	76	34	68	102
10	47	53	100	50	43	93
11	63	31	94	37	55	92
12	35	28	63	52	44	96
13	47	50	97	48	44	92
14	30	37	67	30	67	97
15	27	68	95	26	72	98
16	45	49	94	28	69	97
17	46	32	78	34	55	89
18	38	64	102	40	64	104
19	32	38	70	85	16	101
20	36	64	100	67	30	97
21	43	39	82	46	32	78
22	45	22	67	44	22	66
23	42	24	66	33	38	71
24	48	28	76	46	54	100
25	32	38	70	22	47	69
26	43	44	87	33	61	94
27	45	40	85	43	17	60
28	48	52	100	51	47	98
29	31	32	63	43	45	88
30	75	12	87	71	24	95
Mean	44	38	82	43	49	92

lymphocytes showing surface staining for IgG, IgM, and IgA in synovial fluid (42%) and blood (41%), and the ranges of values obtained were of the same order of magnitude in the case of IgG and IgM. However, the mean percentage of IgA-bearing lymphocytes was significantly higher ($P < 0.01$) in synovial fluid (8%) when compared with blood (5%). The percentage of lymphocytes bearing surface Ig was of the same order of magnitude in the blood and

Table III Percentage of immunoglobulin-bearing lymphocytes (B-cells) in blood and synovial fluid of 30 rheumatoid patients

Immunoglobulin class detected by conjugate	Percentage immunoglobulin-bearing cells				Significance of difference of means
	Blood		Synovial fluid		
	Mean	Range	Mean	Range	
Polyvalent Ig	44	27-75	43	20-85	P > 0.05
IgG	23	14-45	21	6-45	P > 0.05
IgM	13	3-30	13	3-26	P > 0.05
IgA	5	2-10	8	2-18	P < 0.01

Table IV *Immunoglobulins G, M, and A in blood and synovial fluid of 30 rheumatoid patients*

Immunoglobulin class	Immunoglobulin (mg/100 ml)				Significance of difference of means
	Blood		Synovial fluid		
	Mean	Range	Mean	Range	
IgG	1458	880–1950	799	340–1100	P < 0.001
IgM	78	37–200	44	21–115	P < 0.001
IgA	378	200–550	202	58–420	P < 0.001

synovial fluid of half of the patients (Table II). There was no correlation between the immunoglobulin levels in blood and synovial fluid and the percentages or absolute numbers of lymphocytes bearing IgG, IgM, or IgA.

The pattern of immunofluorescent staining of synovial fluid lymphocytes did not differ from that of peripheral blood lymphocytes. The predominant pattern was one of spots uniformly distributed in a ring pattern: less frequently the fluorescence formed a cap at one side of the lymphocyte; and occasionally there was a smooth confluent ring of fluorescence around the periphery of the cell.

COMBINED ROSETTING AND IMMUNOFLUORESCENT STUDIES

In the combined test, carried out in 6 cases, cells showing surface staining for the presence of IgG, IgM, or IgA did not form rosettes, and rosette-forming cells were not stained with polyvalent Ig, IgG, IgM, or IgA conjugates. There was no reduction in rosette-forming cells in preparations in which the lymphocytes had been pre-incubated with conjugates when compared with lymphocytes not pre-incubated with conjugates.

IMMUNOGLOBULIN LEVELS IN SYNOVIAL FLUID AND BLOOD

The mean levels of IgG, IgM, and IgA in the blood were significantly higher ($P < 0.001$ in all cases) than in the synovial fluid, though the levels extended over a wide range with each class of immunoglobulin (Table IV). In both blood and synovial fluid the mean level of IgG was significantly greater than the level of IgA, and the level of IgA was significantly greater than that of IgM ($P < 0.001$ in both cases).

As synovial fluid is so difficult to obtain from normal joints in life, we determined the immunoglobulin levels in 8 synovial fluids obtained from apparently normal knee joints at autopsies on non-rheumatoid subjects performed less than 12 hours after death (Table V). The mean levels of IgG, IgM, and IgA in the autopsy synovial fluid were significantly lower than in the rheumatoid fluids ($P < 0.001$) in all cases.

Table V *Immunoglobulins G, M, and A in synovial fluid of 8 nonrheumatoid 'control' subjects*

Immunoglobulin class	Immunoglobulin (mg/100 ml)	
	Mean	Range
IgG	275	140-740
IgM	28	10-64
IgA	53	10-130

There was no correlation between the level of each class of immunoglobulin and the absolute number or percentage of IgG-, IgM-, or IgA-bearing lymphocytes in the blood or synovial fluid.

Discussion

The techniques used in this investigation are considered capable of distinguishing two distinct subpopulations of lymphocytes in human peripheral blood—the B-cells which possess demonstrable surface Ig determinants and which do not form spontaneous rosettes with SRBC, and the T-cells which form spontaneous rosettes with SRBC and which do not possess demonstrable surface Ig determinants when examined by current techniques of immunofluorescence. In the present study we have applied these techniques to the study of lymphocytes in the peripheral blood and synovial fluid in rheumatoid subjects.

The mean percentage of T-cells in the synovial fluid was significantly higher than in the peripheral blood, though a wide range of values was observed in the group as a whole. Other studies have yielded results showing that the percentage of T-cells in rheumatoid synovial fluid is of the same order of magnitude as in the peripheral blood (Winchester, Bentwick, and Kunkel, 1973; Keith and Currey, 1973; McGill, 1973).

Despite the differences in the mean percentages of T-cells, there was no significant difference between the mean total percentage of B-cells in the peripheral blood and synovial fluid, and a wide range of values was observed in the group as a whole. We were unable to confirm the findings of Winchester and

others (1973) that joint fluids have a relatively lower percentage of B-cells when compared with peripheral blood. An unexpected finding in our study was the significantly higher proportion of synovial fluid lymphocytes bearing surface IgA, and the reason for this remains obscure.

The combined total percentage of identifiable T + B cells was usually in excess of 75%, and exceeded 90% (occasionally achieving 100%) in over two-thirds of the synovial fluids and in nearly half of the blood samples. We are not yet in a position to judge whether the deficits in the combined T + B cell percentages observed by ourselves (in some cases) and others are due to deficiencies in the techniques used, or whether they are due to the presence in some cases of 'null cells' which do not exhibit the known characteristics of T or B cells. The existence and identity of 'null cells' remains a matter of speculation, but it may be significant that improvements in the techniques of rosetting and immunofluorescence in recent years have been accompanied by progressive increases in the percentages of identifiable T and B cells. However, it is of interest that we have found a significantly higher proportion of 'null' cells (indistinguishable from small lymphocytes) in the blood (18%) when compared with synovial fluid (8%): the reasons which underlie this are speculative at the present time. It must also be borne in mind that cell lines not directly related to the T and B lymphocytes may at some stage in their development be morphologically indistinguishable from the small lymphocyte by light microscopy (Vernon-Roberts, 1969).

While the present study shows that abundant T and B cells are present within rheumatoid synovial effusions, it does not show whether these cells are in

whole or in part derived from precursor cells situated within the synovial tissues or whether they selectively migrate to the joint from the blood stream. The higher percentage of T-cells in the synovial fluid could relate to the production of soluble factors for the mediation of local delayed-type hypersensitivity by sensitized T-cells in rheumatoid joints in response to specific antigenic stimuli; or it may relate to the known tissue-damaging activity of some sensitized T-cells for which synovial cells, articular cartilage, or other cells could be the 'target'.

Summary

Using the techniques of immunofluorescent staining for the presence of surface membrane immunoglobulin and spontaneous rosette formation with sheep erythrocytes, B and T cells have been identified in lymphocyte populations obtained from the peripheral blood and synovial fluid of 30 rheumatoid patients. The majority of blood and synovial fluid lymphocytes exhibited either fluorescent staining of surface membrane immunoglobulin or spontaneous rosette formation, and there was no overlap between these phenomena. The mean percentage of rosette forming cells (T-cells) was higher in synovial fluid. Although there was no significant difference between the percentage of cells bearing surface immunoglobulin (B-cells) in blood and synovial fluid, a higher proportion of synovial fluid lymphocytes had surface membrane IgA.

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Addendum

At the International Symposium on Immunological Aspects of Rheumatoid Arthritis held at Montpellier in March 1974, Winchester, Wernet, Fu, and Kunkel presented evidence that surface immunoglobulin demonstrable by immunofluorescence may be due in part to the presence of antilymphocyte antibodies. This antilymphocyte antibody was shed on overnight culture at 37°C leaving a single Ig class on the cell surface in contrast to the polyvalent situation existing on some cells before incubation. Whereas 80–90% lymphocytes showed some surface Ig before incubation, only 4–8% cells possessed surface Ig after incubation. Re-incubation with autologous serum restored the surface Ig to a high percentage of cells. We cannot confirm the findings of Winchester and others, since in our studies of combined rosetting and immunofluorescence rosette-forming cells never possessed surface Ig; moreover, we have not seen a reduction of cells bearing surface Ig after incubation at 37°C. However, it seems that antilymphocyte antibodies are more readily detectable in SLE, and we did not include any SLE patients in our study.

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