Characterisation of activated lymphocytes in the peripheral blood of patients with rheumatoid arthritis

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SUMMARY The extent and nature of lymphocyte activation in the circulation in rheumatoid arthritis (RA) was investigated. Peripheral blood lymphocytes (PBL) from RA patients and healthy controls were separated into a number of discrete fractions by density in discontinuous Ficoll density gradients. Low density (activated) lymphocytes were found at significantly higher levels in RA, particularly in patients with clinically active disease. Conversely, patients with clinically inactive RA had normal levels of activated lymphocytes. Lymphocyte populations within the Ficoll gradient fractions were detected by E rosettes, staining for surface Ig, and by different avidities of EA binding. The activated population in RA was shown to be relatively depleted of T cells, enriched in surface Ig-bearing lymphocytes, and depleted of lymphocytes with high avidity EA binding. The evidence suggests that many of the activated PBL in RA are B blasts.

The elegant thoracic duct drainage (TDD) studies of Paulus *et al.*¹ have suggested an important role for circulating lymphocytes in the immunopathogenesis of rheumatoid arthritis (RA). These authors attributed the clinical improvement observed in their patients to suppression of the immune response following TDD. However, Ueo *et al.*² have shown that the clinical improvement resulting from TDD may be due to an imbalance of the circulating and marginated pools of lymphocytes resulting in an accelerated migration of lymphocytes from inflamed sites in synovial tissue to the blood stream which regresses once TDD is discontinued. Thus, a role for circulating lymphocytes in the pathogenesis of RA has not been proved unequivocally by lymphatic drainage studies. Moreover, this issue is complicated by the varying recirculatory abilities of the different cell types resulting from activation of lymphocytes. While small lymphocytes may recirculate freely, very few, if any, blast cells or plasma cells recirculate from blood to lymph.³ Such activated lymphocytes may be of particular interest in RA, as they either constitute or become the primary effector cells of the immune response and have been found at high levels in the synovial

membrane,⁴ synovial fluid,⁵ and peripheral blood of rheumatoid patients.^{6,7} Circulating blast cells will incorporate increased amounts of ³H-thymidine,⁸ and their levels are also associated with disease activity.⁶

The nature and function of the circulating blasts in RA have not been elucidated. This is largely due to the relatively low percentage of these cells within the total lymphocyte population. Activated lymphocytes in the rheumatoid synovium have received more attention, and it has been shown that, although most RA synovial membrane lymphocytes are T cells,⁹ a disproportionately high number of synovial blasts are B blasts and plasma cells.¹⁰

Studies of lymphocyte subpopulations in the blood have looked at the T and B cell markers¹¹⁻¹³ or Fc receptors^{14,15} of the whole lymphoid population without attempting to analyse the nature of the activated lymphocytes. To this end a means of enriching populations of lymphocytes activated *in vivo*, utilising their decreased density in Ficoll gradients, has been developed¹⁶ and employed in this study to permit further analysis of peripheral blood blasts in RA.

Patients and methods

Forty-seven patients with definite RA were studied (28 females and 19 males) who were receiving non-steroidal anti-inflammatory therapy or analgesics.

Accepted for publication 2 July 1980

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Patients who were receiving, or had been receiving during the month prior to testing, steroids, cytotoxic drugs, or penicillamine were excluded. Clinical assessments of disease activity were made by an independent observer. Thirty-two age- and sex-matched healthy subjects were also studied.

DENSITY FRACTIONATION OF LYMPHOCYTES

Density gradient separation of blood lymphocytes was performed as previously described.¹⁶ Briefly, mononuclear leucocytes were prepared from peripheral blood by Ficoll/Paque separation,¹⁷ the monocytes depleted by adherence to glass, and the remaining lymphocytes (>99%) separated in discontinuous Ficoll (16–30%) density gradients at 3000 *g*. The interfaces of the gradient layers were harvested and the cells washed and counted. The cells in each fraction were expressed as a percentage of the total number recovered from the gradient. Lymphocytes from patients and age- and sex-matched controls were separated in parallel gradients.

E ROSETTE FORMING CELLS

Lymphocytes from the gradient interfaces were washed 3 times with phosphate-buffered saline (PBS), adjusted to $2 \times 10^6 \text{ ml}^{-1}$, and 200 μl of this suspension were mixed with 200 μl washed sheep red blood cells (SRBC) containing 10% new born calf serum. After 10 min incubation at 37°C the cells were pelleted at 200 *g* and incubated at 4°C. The rosettes were resuspended on a rotary turntable and the lymphocytes stained with crystal violet (0.2%) before microscopical examination in a Neubauer chamber. A rosetting lymphocyte was counted as one binding 3 or more SRBC.

SURFACE Ig BEARING CELLS

Peripheral blood lymphocytes were prepared by isopycnic centrifugation and washed 3 times with PBS. Monocytes were removed by incubation at 37°C for 45 min over glass. This procedure also removed labile immunoglobulin on the cell surface. Membrane Ig was stained by incubation with FITC-conjugated antihuman gammaglobulin (Wellcome) at 4°C for 60 min. Three washes in PBS were followed by density separation in a Ficoll gradient. Gradient interface fractions were harvested and washed, and the percentage of fluorescent-labelled cells was counted in a Leitz UV microscope.

FC RECEPTOR BEARING CELLS

These cells were detected by forming rosettes with ox red blood cells (ORBC) coated with rabbit IgG anti-ORBC. The IgG fraction of rabbit anti-ORBC had been purified by affinity chromatography to

protein A-Sepharose CL-4B and subsequent elution with 1 M acetic acid. Equal volumes of washed ORBC (2%) in PBS and IgG antibody at titres of 1/800 or 1/3200 were mixed and incubated at 37°C for 60 min. The ORBC were washed 3 times with PBS, made up to 1%, and equal volumes (200 μl) of coated ORBC and washed gradient interface lymphocytes were mixed, pelleted, and incubated at 4°C for 60 min. EA rosettes were determined as for E rosettes.

STATISTICS

Where applicable the data were analysed for statistical significance by Student's *t* test (with Yates's correction).

Results

DENSITY DISTRIBUTION OF LYMPHOCYTES

Comparisons of density distribution patterns of blood lymphocytes in RA and controls are shown in Fig. 1a. In both RA and controls the majority, 51% and 67% respectively, of the cells were found in dense fraction G. RA patients had significantly more cells than controls in the low density fractions C, E ($P < 0.01$), and D and F ($P < 0.001$) and less in G ($P < 0.001$).

The relationship between lymphocyte density distribution and RA disease activity was investigated. Fig. 1b shows the results from groups of clinically active ($n = 11$) and inactive ($n = 12$) patients. Those with clinically active RA had significantly ($P < 0.001$) fewer lymphocytes (39%) in fraction G than those with inactive RA (65%), with a result that more low density lymphocytes were found in the clinically active patients, notably in fractions F ($P < 0.001$) and C and E ($P < 0.02$). In fact the patients with clinically inactive RA had distribution patterns very similar to those of healthy subjects.

LYMPHOCYTE SURFACE MARKERS IN FICOLL GRADIENT FRACTIONS

Within each set of data in this section the figures represent the percentage positive cells in each gradient fraction. In some subjects there were not enough lymphocytes in the low density fractions for satisfactory surface marker analysis, and hence individual numbers in each experiment are not given. There were usually 10–20 samples assayed per gradient fraction with the exception of fraction A.

The percentage E rosetting lymphocytes in the gradient fractions are shown in Fig. 2a. It may be seen that more of the dense cells (fraction G) form E rosettes in both RA patients and controls, 55% and 62% respectively, whereas in fraction B only

1a

2a

1b

2b

Fig. 1 Lymphocyte density distribution patterns in RA. (a) Health and RA. (b) Clinically active and inactive RA.

30% and 31% of the cells form E rosettes. There were no statistically significant differences between rheumatoid patients and controls.

The B cell marker, integral membrane immunoglobulin (SIg), shows an opposite trend (Fig. 2b),

Fig. 2 Lymphocyte surface markers in Ficoll gradient fractions in health and RA. (a) T cells (E-rosettes). (b) B cells (SIg).

the low density cells having the highest percentages of positively staining cells with decreasing occurrence in the denser fractions. There were no statistically significant differences between RA and controls.

The use of two different IgG concentrations for coating the ORBC in the EA rosetting assay enabled examination of 2 avidities of IgG-FcR binding. At

Fig. 3 EA binding in Ficoll gradient fractions in health and RA. (a) Low + high avidity binding (1/800 titre of coating IgG). (b) High avidity binding (1/3200 titre).

an IgG titre of 1/800 the assay detects high and low avidity binding whereas at 1/3200 only high avidity binding is detectable.¹⁵ Assaying low + high avidity binding within the gradient fractions (Fig. 3a) showed no specific trend in RA or controls, though there is a marked drop in the percentage EA rosettes formed in the dense fraction G by comparison with

fraction F in controls ($P < 0.02$) and, particularly, in RA ($P < 0.005$).

High avidity EA rosette formation, when 1/3200 titre of IgG anti-ORBC was used, produced as expected far fewer rosettes (Fig. 3b). These lymphocytes were found predominantly in the dense cell fractions of the Ficoll gradient. Rheumatoid patients had significantly more rosetting lymphocytes than controls in fractions E ($P < 0.05$), F ($P < 0.10$), and G ($P < 0.01$).

Discussion

In this study we have employed a technique which has enabled us to detect and isolate low density lymphocytes from peripheral blood in RA. There is much evidence from animal experiments that low density lymphocytes contain antigen-activated populations of cells,^{18,19} and we have confirmed a decrease in density in relation to activation of human circulating lymphocytes.¹⁶ The increased incidence of low density lymphocytes in RA is indicative of increased numbers of activated lymphoid cells in the circulation, supporting the reports of increased immunoblast levels by Bacon *et al.*⁶ and Delbarre *et al.*⁷ A decrease in cell density is one of the earliest physical changes associated with lymphocyte activation²⁰ and is directly related to changes in the lymphocyte cytoplasmic refractive index in RA,²¹ another indicator of recent cell activation.²²

The association between disease activity in RA patients and increased numbers of low density lymphocytes is in agreement with reports correlating high levels of immunoblasts in the circulation with clinically assessed disease activity⁶ and high erythrocyte sedimentation rate (ESR).⁷ We have made serial studies of lymphocyte density patterns in rheumatoid patients during treatment with gold and cyclophosphamide, and this appears as a separate publication.²³

Whether the blasts divide, die, or develop in the circulation or spread the immune response to other tissues, they must be produced in large numbers, as their life in the circulation is only hours²⁴ in comparison with the months that small lymphocytes may survive.²⁵ The site of activation may be lymph node(s), as is frequently the case in antigenic triggering of proliferation, or synovial tissue itself, which, notably in RA, has many of the characteristic properties of secondary lymphoid tissue.²⁶ No specific antigen has been successfully identified within the rheumatoid joint, though antigens may be sequestered in central or peripheral lymphoid tissue.

The predominance of B cells in the activated populations in RA which we have shown in this

study corresponds with the low incidence of T cells in these fractions. The assay employed here for surface Ig detection would not have detected cytophilic IgG attached to Fc receptors²⁷ or plasma cells in the later stage of their development, since these lose their surface Ig,²⁸ and thus the activated populations may contain more B cells than reported. Stimulation of purified T cells with allogeneic lymphocytes produces no change in the detection of receptors for SRBC,^{29 30} which suggests a true representation by the T cell levels reported in this study.

The increased EA binding in RA at low concentrations of erythrocyte coating IgG is in accordance with the data of Sharpin and Wilson,¹⁴ implying either increased numbers of Fc receptors or the presence of Fc receptors with high avidity binding to complexed IgG. Our observations of high avidity EA binding in the denser fractions, containing exclusively small lymphocytes, is suggestive of K cell activity in these fractions.³¹ As K cells are non-B,³² this could account for the distribution of high avidity Fc binding within the Ficoll gradient fractions.

We propose that in RA there is increased B cell activation of the blood lymphocytes and that there are increased numbers of non-B, non-T lymphocytes possessing increased Fc-binding. Both abnormal populations could contribute to the pathology and persistence of joint damage in RA.

We are grateful to Anne Willington for secretarial assistance and to Ciba-Geigy for financial support (SDC).

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