Morphological evidence that activated polymorphs circulate in the peripheral blood of patients with rheumatoid arthritis

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Abstract

Purified peripheral blood polymorphonuclear leucocytes (PMNs) from patients with rheumatoid arthritis (RA) have been found to differ from purified PMNs from normal subjects in ways that are consistent with their prior activation. However, it is currently contentious whether activated PMNs really circulate in patients with RA, or whether they are produced as an in vitro artefact of purification. Recently developed rapid leucocyte fixation and preparation technique showed that the proportion of polarised (activated) PMNs (36·9 (24·7)%, mean (SD); $n=31$) was increased relative to that in control subjects (8·1 (5·6)%; $n=12$). Serum cytidine deaminase levels, a biochemical marker of PMN lysis, were also increased in patients with RA (11·59 (7·26) U/ml) compared with those in controls (6·82 (3·78) U/ml), but the proportion of polarised PMNs and the levels of cytidine deaminase activity were unrelated to clinical assessments of inflammatory disease activity.

Twelve patients who were not receiving drugs or who were receiving only non-steroidal anti-inflammatory drugs (NSAIDs) had more polarised PMNs than 19 patients receiving second line treatment in addition to NSAIDs (patients receiving NSAIDs, 49·6 (25·9)%; patients receiving second line treatment, 27·5 (21·1)%). Fluorescence activated cytometric analysis of CR1 and CR3 expression on PMNs from a randomly selected subgroup of patients with RA showed that the serum level of cytidine deaminase activity was correlated positively with the expression of CR1 (the C3b receptor) on the cell surface and that the proportion of polarised PMNs was positively correlated with the expression of CR3 (or CD11b/CD18), the iC3b receptor that is upregulated on activation. It is suggested that the polarised PMNs which circulate in blood samples from patients with RA represent cells which have been activated but not yet marginated, or activated cells which have marginated but subsequently returned to the circulating pool.

Preparations of peripheral blood polymorphonuclear leucocytes (PMNs) from patients with rheumatoid arthritis (RA) have frequently been found to contain cells which show evidence of prior activation.[1 2] For example, preparations of PMNs from patients with RA contain more cells with a low electrophoretic mobility[3] and a polarised (non-spherical) shape[4] than do similar preparations from control subjects. It is surpris-

ing that substantial numbers of activated PMNs can be recovered from patients with RA by venepuncture, because if normal PMNs are activated in vitro, they show an increased adherence to cultured endothelial cells[5] and the infusion of activators, for example, C5a, into animals in vivo causes a transient neutropenia that is attributed to increased margination.[6] It is possible that the differences seen between purified PMNs from patients with RA and control subjects might possibly be an artefact due to selective changes in vitro during the purification procedure, as blood samples from patients with RA often contain substances such as immune complexes for example, that are capable of eliciting similar changes in normal PMNs, for example, decreases in electrophoretic mobility.

One way to resolve this issue might be by immediately fixing PMNs in blood samples ex vivo and subsequent examination. A convenient indicator of activation in this respect is cell shape, as it has been shown that the polarising responses in PMNs that are elicited by chemotactic agents in vitro can be easily monitored by light microscopy.[8] Moreover, a rapid leucocyte fixation and preparation technique has recently been developed specifically for this purpose.[9] We have used this technique to look for morphological evidence of PMN activation in blood samples of patients with RA and control subjects. In addition, we have assessed the activity of cytidine deaminase (CD) in serum samples and have also examined the surface expression of CR1 and CR3 on PMNs in subgroups of patients and controls, as these parameters might be expected to correlate with the extent of PMN activation.

Patients and methods

PATIENTS AND CONTROLS

Thirty one patients (22 women, nine men, mean age 56 years, range 28–81 years) who had classical or definite RA (American Rheumatism Association criteria) were studied. Inflammatory disease activity was assessed on a scale of 0–3 (0=none, 3=maximum) by a combination of the duration of early morning stiffness, joint activity, an articular index, laboratory parameters where available (erythrocyte sedimentation rate, C reactive protein, white blood cell count) and a pain score. Two patients were classified as grade 0, 14 as grade 1, nine as grade 2 and six as grade 3. Four patients were receiving no drugs and all of the others were receiving NSAIDs. In addition, eight were receiving penicillamine, four cytotoxic drugs, five steroids and cytotoxic drugs, three steroids

and penicillamine, one penicillamine and a cytotoxic drug, one gold salts and one chloroquine. Twenty two patients were latex positive; 12 had extra-articular disease.

The control subjects were healthy individuals (four women, eight men, mean age 37 years, range 21–55 years) who were receiving no drugs and who had no known inflammatory conditions.

All patients and controls gave their informed consent for the study.

LEUCOCYTE PREPARATION AND SHAPE ANALYSIS OF PMNS

The leucocytes were prepared for light microscopy as described previously.[9] Briefly, 1 ml venous blood drawn from the median antecubital vein was immediately mixed with 1 ml pre-warmed (37°C) phosphate buffered saline (PBS) or Hanks's buffered salt solution with 0·01 M Hepes pH 7·3 buffer (HHBSS) containing 0·4% formaldehyde and kept for 4 min at 37°C. Erythrocytes were lysed by the addition of 40 ml 0·155 M ammonium chloride pH 7·3 (or in some instances 0·155 M ammonium chloride buffered with 0·01 M Tris chloride or 0·01 M Hepes, pH 7·3). Leucocytes were collected by centrifuging at 200 g for 5 min, washed twice by centrifuging at 200 g for 10 min in PBS or HHBSS (40 ml and 20 ml consecutively), resuspended in 1 ml PBS or HHBSS and stored at 0°C. Preparations were mixed (1:1) with toluidine blue (0·01% in PBS) and the cells allowed to settle by gravity onto poly-L-lysine coated cover slips. When the cells had adhered, the cover slip was placed inverted on a cavity slide and examined using a Leitz microscope with bright field illumination and an oil immersion (×70) objective lens. Photomicrographs were taken with a Leitz Dialux 20 microscope.

Neutrophils were assigned by one of three observers (who, whenever possible, were blind to the diagnosis) into one of the following categories on the basis of shape: spherical, slightly polar, moderately polar or strongly polar.[9] At least 150 well separated cells were counted in each preparation and the percentage score was calculated for each category. Altogether a total of 17 leucocyte preparations were made on different occasions from the 12 control subjects (five from one subject, two from another, and one each from the remainder), and one preparation from each of the patients with RA. Whenever practicable, a blood sample from a control subject was processed alongside a blood sample from one or more patients with RA.

DETERMINATION OF SURFACE EXPRESSION OF CR1 AND CR3

Samples (25 µl) of the leucocyte preparations from a randomly selected subgroup of the patients with RA ($n=14$) and control subjects ($n=12$) were labelled within two hours with monoclonal antibodies kindly supplied by Professor P C Beverley, Professor W Bodmer, Dr N Hogg and Professor A McMichael, which were either ascites fluid that had been titrated to give a working dilution (usually 1:1000) or undiluted culture supernatants (table). Incubations were performed on a microtitre plate (Falcon 3911 Microtest III, Becton Dickinson, Cowley, Oxford, UK) at 0°C for 30 minutes and the cells were washed after each incubation with 100 µl of medium. RPMI medium containing 5% fetal calf serum was used throughout. Controls cells were prepared which were either incubated with mouse monoclonal antibody alone or with a subclass matched irrelevant monoclonal antibody (UCHTI). Fluorescein conjugated rabbit anti-mouse (Fc F(ab)$_2$) diluted 1:50 (Dakopatts, Weybridge, Surrey, UK) was used as the second layer. Labelled cells were resuspended in 25 µl of RPMI fetal calf serum, transferred to round bottomed polystyrene tubes containing 1% paraformaldehyde, then stored for two hours in the dark at 40°C and analysed by flow cytometry using a FACscan (Becton Dickinson, Mountain View, CA, USA) equipped with Consort 30 software (Hewlett Packard). The polymorphs were separated on the basis of their forward and side scatter; their identity was verified by staining with monoclonal antibodies which identify leucocytes (W6/32 and 2D1) and leucocyte subpopulations (29 and TG1). Specificity was verified with a subclass matched control antibody (UCHT1). Fluorescence data were collected on a four decade logarithmic scale; markers were set on the control antibody (UCHT1). There was generally less than 5% non-specific staining. The mean fluorescence intensity ratio was calculated by dividing the mean fluorescence intensity of the polymorph population labelled with a given monoclonal antibody by the mean fluorescence intensity of the same population labelled with the negative control antibody. These experiments formed part of a larger study of leucocyte integrin and CR1 surface expression

Monoclonal antibodies used in indirect fluorescence cytometry

Monoclonal antibody	CD antigen	Cell specificity	Isotype	Reference
W6/32[a]	—	All leucocytes (HLA,A,B,C)	IgG1	10
2D1[a]	CD45	All leucocytes	IgG1	11
29[b]	CD15	Polymorphs, monocytes, natural killer cells	IgM	12
TG1[b]	Unknown	Polymorphs, monocytes	IgM	12
UCHTI[c]	CD3	T lymphocytes	IgG1	13
MHM23[d]	CD18	All leucocytes	IgG1	14
44[b]	CD11b	Polymorphs, monocytes, natural killer cells (CR3)	IgG1	15
E13[b]	CD35	Polymorphs, monocytes Lymphocytes (CR1)	IgG1	16

[a] Supplied by Professor W Bodmer.
[b] Supplied by Dr N Hogg.
[c] Supplied by Professor P C Beverley.
[d] Supplied by Professor A McMichael.

in patients with RA and controls which will be presented elsewhere.

MEASUREMENT OF CD ACTIVITY

A portion (4 ml) of the blood collected by venepuncture was allowed to clot at 37°C for one hour. Serum was separated by centrifugation, frozen within two hours and kept at −20°C until assayed. The CD activity was estimated in samples obtained from all but one of the patients with RA and all but one of the controls, essentially as described previously,[17] except that the samples were incubated with cytidine at 37°C for four hours. This modification yields estimates of CD activity which are routinely 3·25 times higher than those published previously.[17]

STATISTICAL ANALYSIS

Differences between the subject groups were compared by the Mann-Whitney test with two-tailed p values. Correlations were made using Spearman's rank correlation test and Pearson's linear regression analysis. p Values less than 0·05 were considered significant when comparisons were being made between groups and values of less than 0·01 were considered significant when multiple correlations were being tested.

Results

In the leucocyte preparations from control subjects, most of the neutrophils were spherical or slightly polarised and the proportions of moderately and strongly polarised cells were always low. Compared with control subjects, the percentages of spherical cells were decreased and those of slightly polarised, moderately polarised and strongly polarised cells were
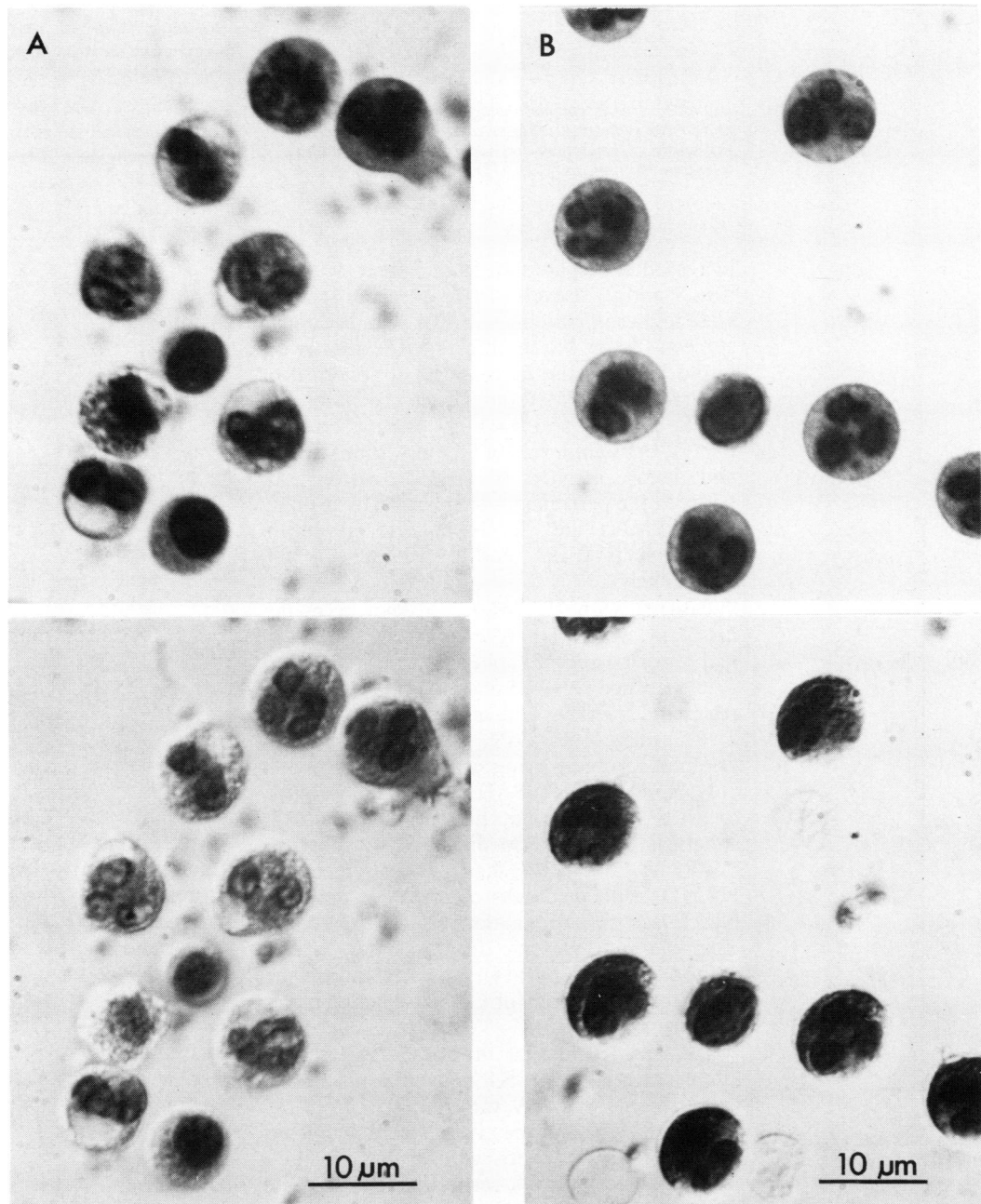

Figure 1   Toluidine blue stained leucocyte preparations from (A) a patient with RA and (B) a control subject. Upper panel: bright field illumination. Lower panel: Normarski differential interference contrast.
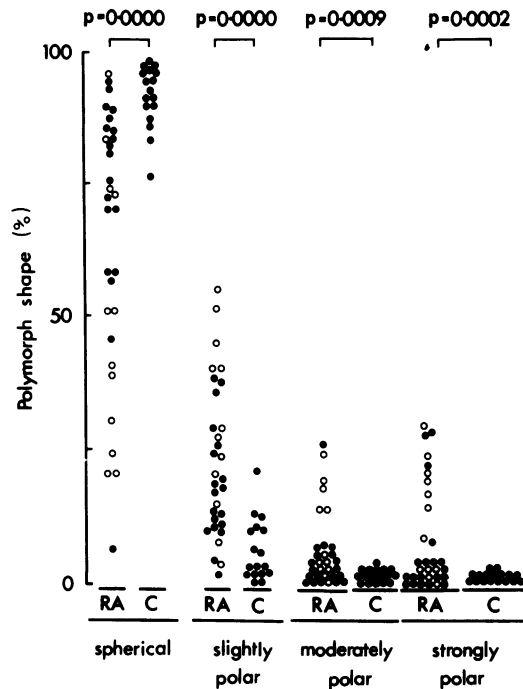
Figure 2 *Polymorph morphology in patients with RA and control subjects (C). Shape categories are as described previously.[9] Open symbols represent those patients with RA who were receiving only NSAIDs or no drugs.*

Figure 4 *Correlation between the levels of serum CD activity and the mean fluorescence intensity ratio of CR1 on polymorphs from patients with RA.*

Figure 3 *Levels of CD in serum from patients with RA, and control subjects (C). Open symbols represent those patients with RA who were taking only NSAIDs or no drugs.*

Figure 5 *Correlation between the percentage of spherical polymorphs and the mean fluorescence intensity ratio of CR3 on polymorphs in patients with RA.*

increased in preparations from patients with RA (figs 1 and 2). Levels of CD in serum samples were higher in patients with RA than in control subjects (fig 3). No significant correlations were found between the percentages of spherical or strongly polar PMNs and disease activity ($r=0.108$, $p=0.60$ and $r=0.08$, $p=0.68$, respectively), between levels of CD in serum samples and disease activity, ($r=0.026$, $p=0.900$), or between the percentages of spherical or strongly polar PMNs and levels of CD in serum ($r=0.244$, $p=0.193$, and $r=0.192$, $p=0.308$, respectively).

Patients with RA who were not receiving drugs, or who were receiving only NSAIDs, had fewer ($p=0.037$) spherical PMNs (median 45.6%, range 20.4–95.8%, $n=12$) than those receiving NSAIDs and other drug(s) (median 75.0%, range 6.9–94.6%, $n=19$). Patients who were not receiving drugs or who were receiving only NSAIDS, or NSAIDs with choloroquine or with penicillamine, had fewer ($p=0.015$) spherical PMNs (median 51.0%, range 6.9–95.8%, $n=17$) than those receiving NSAIDs with gold salts or cytotoxic drugs or NSAIDs with combinations of steroids, penicillamine and cytotoxic drugs (median 77.8%, range 57.0–94.6%, $n=14$). No correlations were found between levels of CD in serum samples and drug treatment.

The mean values of the mean fluorescence intensity ratios for CR1 and CR3 on PMNs from patients with RA (mean (2SD) 1.76 (0.29) and 3.70 (0.62), respectively) were increased slightly compared with control PMNs (1.71 (0.34) and 2.88 (0.67), respectively), but these differences were not considered significant ($p=0.682$ for CR1 and $p=0.085$ for CR3). Linear and rank correlation analysis showed that for
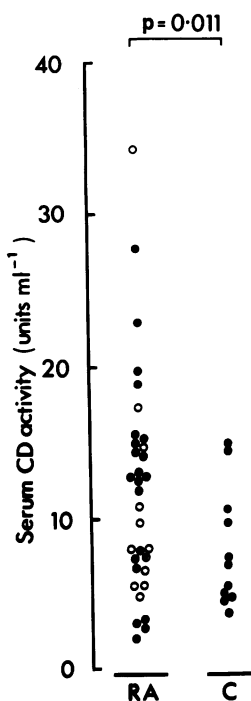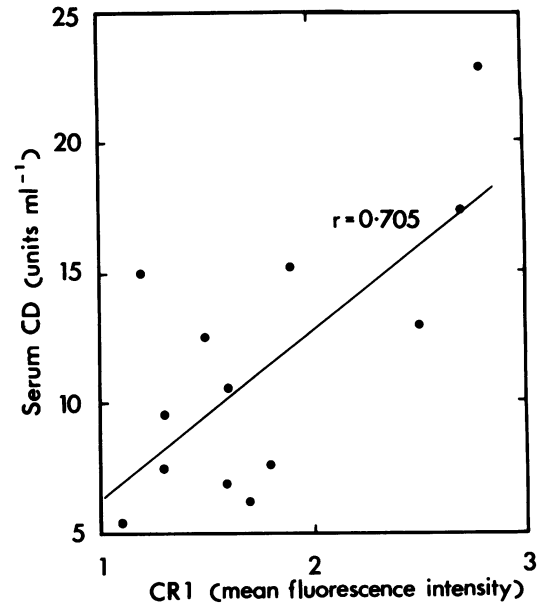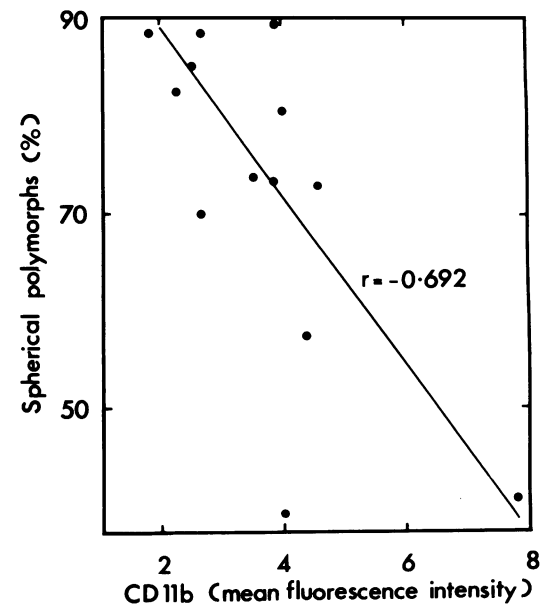
PMNs from patients with RA, the mean fluorescence intensity ratio of CR1 was positively correlated with the activity of CD in serum samples ($r=0.705$, $p=0.007$; $\varrho=0.590$, $p=0.01$) (fig 4) and that the mean fluorescence intensity ratio of CR3 was negatively correlated with the percentage of spherical cells ($r=-0.692$, $p=0.009$; $\varrho=-0.575$, $p<0.025$) (fig 5). Also for this subgroup of patients, the disease activity was weakly correlated with CD activity in serum ($r=0.597$, $p=0.031$; $\varrho=0.461$, $p<0.1$).

Discussion
Except for the unlikely possibility that changes occurred selectively in blood samples from patients with RA during venepuncture, our

results provide unequivocal evidence that the morphology of peripheral blood PMNs from patients with RA differs from that of PMNs from control subjects. Moreover, the range of morphology seen among PMNs from patients with RA resembles that seen in normal PMNs when blood samples have been incubated with chemotaxins in vitro.[9] As polarisation is just one of many responses which PMNs make when activated by chemotaxins in vitro,[18 19] the simplest explanation of these results is that the polarised cells seen in blood samples from patients with RA represent a subpopulation of cells which have been activated by chemotaxins in vivo. In addition, there is ample evidence for the increased production of activated complement components and inflammatory cytokines in patients with RA.[20-22]

Substantial increases in the surface expression of CR1 and CR3 occur rapidly when PMNs are exposed to chemotaxins in vitro,[23 24] but only insignificant increases were found on PMNs from patients with RA. Although this result does not support the contention that chemotaxin activated PMNs can be found in the circulating leucocyte pool of patients with RA, it should be remembered that the concentration of chemotaxins required to elicit secretory functions in PMNs is an order of magnitude higher than that needed to elicit chemotactic functions. For instance, the concentration of formyl methionyl-leucyl-phenylalanine required for half-maximum increases in CR3 expression is $1 \cdot 25 – 2 \cdot 5 \times 10^{-9}$ mol/l,[23] whereas concentrations of 7×10^{-11} and 1×10^{-10} mol/l elicit half-maximal responses in chemotactic migration[25] and actin polymerisation,[26] respectively. Thus shape changes are likely to be a more sensitive indicator of PMN activation in vivo than are increases in the surface expression of CR1 and CR3. Interestingly, in this subgroup of patients with RA there were correlations between serum CD activity and CR1 expression on PMNs, between CR3 expression and PMN shape, and between levels of CD in serum samples and disease activity, the last of these having been noted previously.[17]

Why PMNs showing morphological signs of activation should occur in the circulating leucocyte pool in patients with RA is, at first, puzzling. However, neutrophil dynamics in experimental models of acute C5a induced neutropenia[6] may differ substantially from those in chronic inflammatory conditions and it is possible that the rate of margination and extravasation at inflammatory foci in patients with RA is limiting. The polarised PMNs found in blood samples from patients with RA may represent cells responding to chemotaxins but whose adherence receptors (principally CR3 or CD11b/CD18) have not been upregulated (for the reasons discussed above), or cells whose CR3 expression and adhesiveness have been increased slightly but which have as yet failed to marginate. It has been found that gp100[MEL 14], a murine adhesion protein involved in neutrophil extravasation during inflammation, and its human homologue, LAM.1, are rapidly down-regulated after activation by chemotactic factors in contrast with CR3 (CD11b/CD18), which is

rapidly upregulated.[27 28] In addition, activated neutrophils with high CD11b/CD18: low gp100[MEL 14] expression are unable to localise to inflamed tissues if injected intravenously.[29] Unfortunately, we were unable simultaneously to determine the expression of LAM.1.

As the number of patients with RA who were receiving a particular drug or combination of drugs was small (between one and eight), only limited conclusions on the effect of the drugs can be drawn. Nevertheless, it seems hardly surprising that higher proportions of polarised (or activated) PMNs were found in patients with RA receiving no drugs or receiving only NSAIDs than were found in patients receiving disease modifying drugs in addition to NSAIDs.

Our results suggest that substantial numbers of PMNs showing morphological signs of activation were circulating in the blood of patients with RA, but that the proportions of these cells found in the circulating pool were unrelated to clinical assessments of inflammatory disease activity. Significant relationships were found, however, between markers of PMN activation (i.e. PMN morphology and the surface expression of CR1 and CR3) and neutrophil lysis (i.e. serum CD activity). Further studies are clearly needed to define better the dynamics of PMN behaviour in chronic inflammatory conditions such as RA.

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