Leucocyte integrin and CR1 expression on peripheral blood leucocytes of patients with rheumatoid arthritis

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Abstract
Expression of the leucocyte integrins (CD11a, b, c/CD18) and of CD35 (CR1) on leucocytes from the peripheral blood of patients with rheumatoid arthritis (RA) (n=14) and control subjects (n=12) was measured by flow cytometry using a rapid fixation and leucocyte preparation procedure. The mean (SE) percentages of lymphocytes expressing CD11a (RA 93·4 (1·7)%; controls 97·2 (1·8)%) and CD18 (RA 91·3 (2·3)%; controls 97·0 (2·6)%) were lower and the percentage of monocytes expressing CD11b (RA 86·9 (11·4)%; controls 78·4 (11·9)%) and CR1 (RA 62·6 (15·5)%; controls 36·6 (17·6)%) were higher in patients with RA than in controls. In addition, the mean fluorescence intensity of CD18 (RA 22·1 (2·3); controls 30·7 (2·5)) on lymphocytes was decreased and that of CD11b (RA 4·5 (0·8); controls 2·9 (0·9)) and CR1 (RA 2·4 (0·4); controls 1·5 (0·5)) on monocytes was increased in patients with RA compared with controls. The functional importance (if any) of the altered expression of the antigens on lymphocytes is not yet known. Altered expression on monocytes is consistent with activation within the circulation.

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by infiltration of mononuclear cells into the synovium and the accumulation of leucocytes (predominantly polymorphs) in the synovial fluid of the affected joints. A crucial early event in all inflammatory responses is the localised increase in the adhesion of leucocytes to endothelial cells[1] and several molecules essential to this interaction have been identified.[2 3] On leucocytes, the best known adhesion molecules belong to the glycoprotein complex CD11/CD18 (Leu-CAM or the leucocyte integrins).[4 5] The CD11/CD18 complex consists of CD11a/CD18, CD11b/CD18, and CD11c/CD18 which have distinct α chains of 175, 165, and 150 kilodaltons, respectively, but share a common β chain of 95 kilodaltons.[4 5] CD11a/CD18 is expressed on almost all leucocytes. It mediates the binding of lymphocytes to endothelial cells via their expression of ICAM 1 and ICAM 2.[4 5] CD11b/CD18 and CD11c/CD18 are not expressed on lymphocytes but are expressed to various extents on monocytes and polymorphs. CD11b/CD18 mediates the binding of phagocytic cells, not only to iC3b coated surfaces, but also to endothelial cells and a variety of other ligands including matrix molecules; it is probably the major adhesion molecule responsible for neutrophil-endothelial cell bind-

ing and neutrophil aggregation. No ligand unique to CD11c/CD18 has yet been identified, but CD11c/CD18, like CD11b/CD18, will bind to iC3b.[4 5]

Substantial amounts of CD11b/CD18 and CD11c/CD18 are stored in intracellular vesicles or granules in polymorphs and monocytes; little CD11a/CD18 is stored intracellularly in any leucocytes. Exposure to inflammatory cytokines or activating stimuli mobilises these intracellular reserves causing a two to twelve fold increase in the surface expression of CD11b/CD18 and CD11c/CD18 on polymorphs and monocytes.[6–9] As inflammatory cytokines and other activating stimuli have long been known to elicit increased adherence of leucocytes to endothelial cells, it has been suggested that increases in the expression of these integrins might be causally related to increased adhesiveness of neutrophils,[6 10] although recent studies have not supported this contention.[11–16]

Largely from the results of in vitro studies, several mechanisms have been described which might operate in vivo to induce leucocytes to leave the circulation and enter the joints or other inflammatory foci in patients with RA. These include upregulation of integrins on leucocytes, which is supported by reports that the expression of CD11a/CD18 on lymphocytes from RA synovium[17] and of CD11b/CD18 on polymorphs from RA synovial fluids[18] is increased. As changes in the expression of integrins on circulating leucocytes before margination and emigration may be an important factor in the pathogenesis of RA, we have examined the hypothesis that the expression of components of the CD11/CD18 complex and CD35 (the complement receptor, CR1) on peripheral blood leucocytes from patients with RA may differ from that of normal control subjects. Furthermore, because it is essential to avoid increasing artefactually the expression of CD11b/CD18 and CD11c/CD18 on monocytes and polymorphs by in vitro preparative techniques,[6 19] we have developed and used a procedure whereby leucocytes are fixed immediately ex vivo before staining their cell surface antigens for analysis by fluorescence activated cytometry.

Patients and controls
Fourteen patients (nine women, five men; mean age 54 years, age range 37–73 years) with classical or definite RA defined by the American Rheumatism Association criteria, who were being treated in the wards (six patients) or attending the outpatient rheumatology clinic (eight patients) of the Royal London Hospital

were studied. The activity of inflammatory articular disease activity was assessed on a scale of 0 to 3 (0=no activity, 3=maximum activity) by a combination of the duration of early morning stiffness, joint activity, an articular index, laboratory parameters (erythrocyte sedimentation rate, C reactive protein and leucocyte count), and pain score. On this basis, eight patients were classified as having grade 1, five grade 2, and one grade 3 inflammatory disease. One patient was receiving no drugs; all of the others were receiving non-steroidal anti-inflammatory drugs. In addition, five patients were receiving steroids, four penicillamine, four azathioprine, two methotrexate, and one had received gold one day before the study began. Seven patients were latex positive (titre >1/80), and five had extra-articular disease.

The controls were healthy subjects (two women, nine men; mean age 31 years, age range 21–50 years) who were receiving no drugs and who had no known inflammatory disease. All patients and controls gave their informed consent before the study.

REAGENTS AND MATERIALS
Dulbecco's phosphate buffered saline without calcium and magnesium ions was obtained from Oxoid (Basingstoke, Hampshire, UK) and electron microscopy grade formaldehyde from Taab (Reading, Berkshire, UK). Hanks's buffered salt solution without calcium and magnesium ions, RPMI 1640 medium and fetal calf serum were obtained from Gibco (Paisley, UK). Fluorescein labelled F(ab)$_2$ fragments of rabbit anti-mouse immunoglobulin Fc region were obtained from Dakopatts (Weybridge, Surrey, UK); TRIS N-2-hydroxyethyl-piperazine-N-2-ethane and all other reagents, which were of AnalaR grade whenever possible, were obtained from BDH (Poole, Dorset, UK). Falcon 3911 Microtest III flexible round bottomed microtitre plates and Falcon 2054 round bottomed polystyrene tubes were obtained from Becton Dickinson (Cowley, Oxford, UK). Monoclonal antibodies were either ascites fluids which had been titrated to give a working dilution (usually 1:1000) or undiluted culture supernatants (table 1).

LEUCOCYTE PREPARATION, LABELLING, AND ANALYSIS
Leucocytes were prepared for labelling using a procedure developed originally for morphological analysis.[21] Briefly, blood (0·5 ml) taken from the antecubital vein was mixed immediately with 0·5 ml of prewarmed (37°C) phosphate buffered saline or Hanks's balanced salt solution with 0·01 mol/l HEPES (pH 7·3) (HHBSS) containing 0·4% formaldehyde and stored for 4 min at 37°C. A 20 ml portion of 0·155 mol/l ammonium chloride buffered with 0·01 mol/l TRIS chloride or 0·01 mol/l HEPES (pH 7·3) was added, and when the erythrocytes had lysed (2–4 minutes at 37°C or 8–12 minutes at 20°C), the leucocytes were collected by centrifuging for 5 minutes at 200 g, washed twice by centrifuging for 10 minutes at 200 g in phosphate buffered saline or Hanks's balanced salt solution (first 20 ml, then 10 ml at 0°C), resuspended in 0·5 ml of phosphate buffered saline and stored at 0°C. Cell samples (25 µl) were labelled within two hours. Incubations were performed in a microtitre plate at 0°C using 30 minute periods and the cells were washed after each incubation with 100 µl of medium. RPMI containing 5% fetal calf serum was used as the medium throughout.

Control cells were prepared which were either incubated with mouse monoclonal antibody alone or with a subclass matched irrelevant monoclonal antibody (PDS1 or BDS4). Fluorescein conjugated rabbit antimouse Fc F(ab)$_2$ diluted 1:50 was used as the second layer. Labelled cells were resuspended in 25 µl of RPMI containing 5% fetal calf serum, transferred to round bottomed polystyrene tubes containing 1% paraformaldehyde, left for two hours in the dark at 4°C and analysed by flow cytometry using a FACscan (Becton Dickinson, Mountain View, CA, USA) equipped with Consort 30 software (Hewlett Packard). Polymorphs, monocytes, and lymphocytes were separated on the basis of their forward and side scatter; their identity and purity were established by staining with monoclonal antibodies which identify leucocytes (W6/32 and 2D1), and leucocyte subpopulations (29, TG1, UCHM1 and UCHT1). Fluorescence data were collected on a four decade logarithmic scale. Markers were set on the control antibody, which was PDS1 for monocytes and lymphocytes and UCHT1 for polymorphs because of the unusually high non-specific binding of PDS1 by polymorphs. There was generally less than 5% non-specific staining. The percentage of fluorescent cells was calculated by subtracting the negative control values from the total in each instance. The mean fluorescence intensity ratio was calculated by dividing the mean fluorescence intensity ratio of a leucocyte population labelled with a given monoclonal antibody by the mean fluorescence intensity ratio of the same population labelled with the negative control antibody.

Table 1 Monoclonal antibodies used in indirect fluorescence for cytometry

Monoclonal antibody	CD antigen	Cell specificity	Isotype	Reference
PDSI*	—	Porcine retina (S-antigen)	IgG1	20
BDS4*	—	Porcine retina (S-antigen)	IgG2a	20
W6/32†	—	All leucocytes (HLA, A, B, C,)	IgG1	22
2D1†	CD45	All leucocytes	IgG1	23
29‡	CD15	Polymorphs, monocytes, natural killer cells	IgM	24
TG1‡	Unknown	Polymorphs, monocytes	IgM	24
UCHM1§	CD14	Monocytes	IgG2a	24
UCHT1§	CD3	T lymphocytes	IgG1	25
MHM23¶	CD18	All leucocytes	IgG1	26
MHM24¶	CD11a	All leucocytes	IgG1	26
3·9‡	CD11c	Polmorphs, monocytes, natural killer cells	IgG1	27
44‡	CD11b	Polymorphs, monocytes, natural killer cells	IgG1	28
E11‡	CD35	Polymorphs, monocytes, N lymphocytes (CRI)	IgG1	29

*Kindly provided by Dr Suleyman.
†Kindly provided by Professor Bodmer.
‡Kindly provided by Dr Hogg.
§Kindly provided by Professor Beverley.
¶Kindly provided by Professor McMichael.

STATISTICAL ANALYSES
ANOVA and the Mann Whitney rank sum test for unpaired data were used to compare data from patients with RA and controls; the two procedures gave essentially similar results. All

calculations, correlations, and multiple regression analyses were performed using the 'number cruncher' statistical package on a personal computer; statistical significance was accepted when $p<0.05$.

Results

The intersubject variation in the proportion of cells expressing a particular surface marker varied greatly among the different markers and leucocyte types. However, figs 1–3 show that the ranges for any given marker and cell type were largely similar for patients with RA and controls, with the exception that the medians for the percentage of lymphocytes expressing CD11a and CD18 were smaller and those for the percentage of monocytes expressing CD11b and CR1 were higher in patients with RA than in controls (figs 1 and 2).

Similarly, the intersubject variation in the values found for the mean fluorescence intensity ratio of a particular surface marker differed greatly among the various markers and leucocyte types. Figures 4–6 show that, again, the ranges for any given marker and cell type were largely similar for patients with RA and controls. However, the median value of the mean fluorescence intensity ratio for CD18 on lymphocytes was decreased and that for CD11b and CR1 on monocytes were increased in patients with RA compared with controls (figs 4 and 5).

Evidence suggesting that a given α chain might be expressed at similar relative levels on different leucocyte types within patients, was found only for CD11a on lymphocytes and polymorphs in control subjects and for CD11b and CD11c on monocytes and polymorphs in controls and patients with RA (table 2). Conversely, evidence suggesting that, within subjects, the different α chains might be expressed at similar relative levels on given leucocyte types, was found only for CD11a and CD11b on control monocytes, RA lymphocytes, and RA monocytes, and for CD11b and CD11c on control and RA monocytes (table 3).
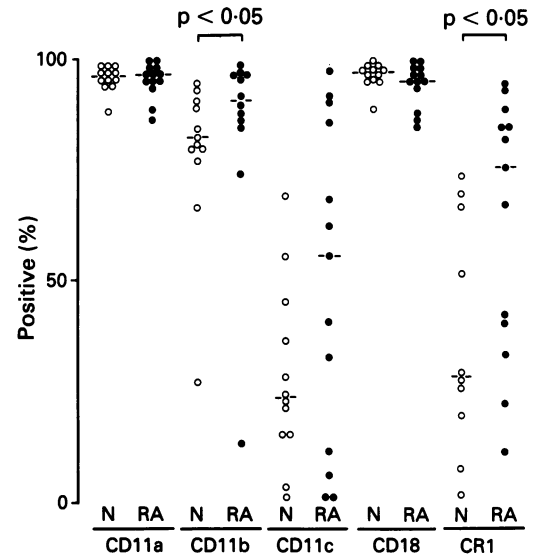
Figure 2   Percentage of monocytes which express CD11/CD18 and CR1. N=control subjects, RA=patients with rheumatoid arthritis. Bars indicate median values.
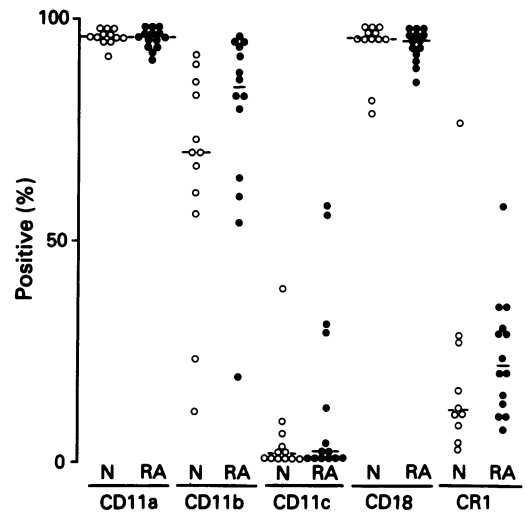
Figure 3   Percentage of polymorphs which express CD11/CD18 and CR1. N=control subjects, RA=patients with rheumatoid arthritis. Bars indicate median values.
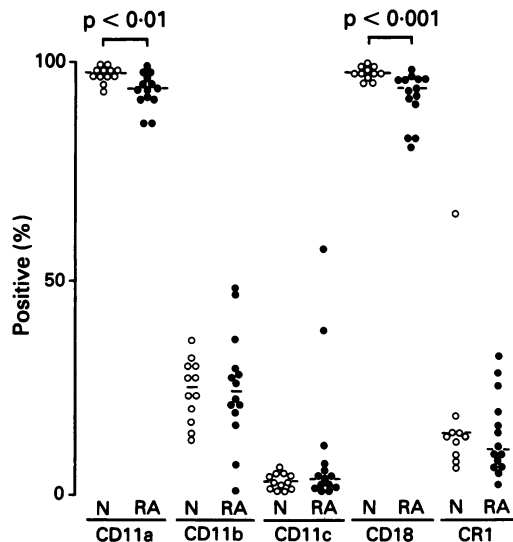
Figure 1   Percentage of lymphocytes which express CD11/CD18 and CR1. N=control subjects, RA=patients with rheumatoid arthritis. Bars indicate median values.
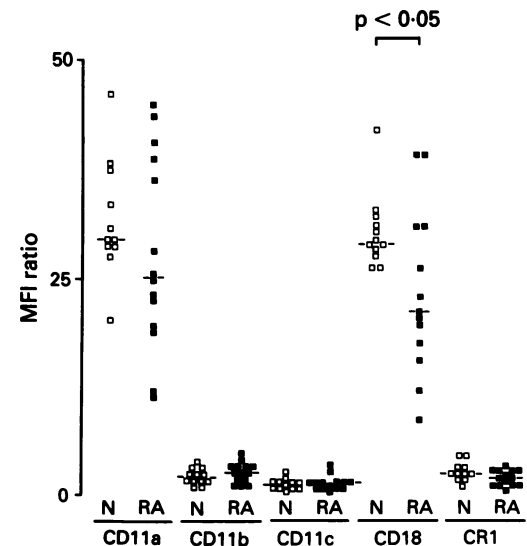
Figure 4   Mean fluorescence intensity ratios of CD11/CD18 and CR1 on lymphocytes from control subjects (N) and patients with rheumatoid arthritis (RA). Bars indicate median values.
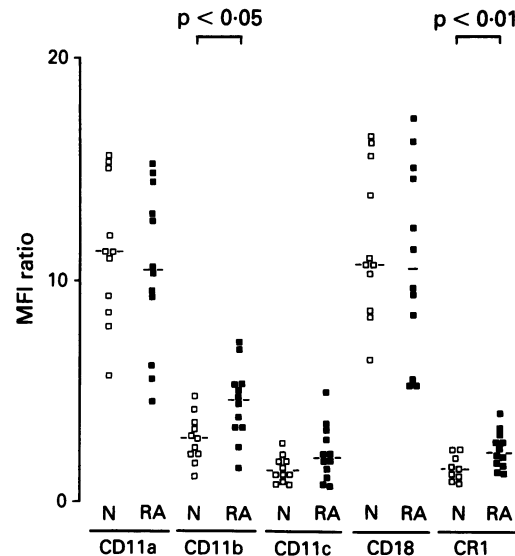
Figure 5   Mean fluorescence intensity ratios of CD11/CD18 and CR1 on monocytes from control subjects (N) and patients with rheumatoid arthritis (RA). Bars indicate median values.
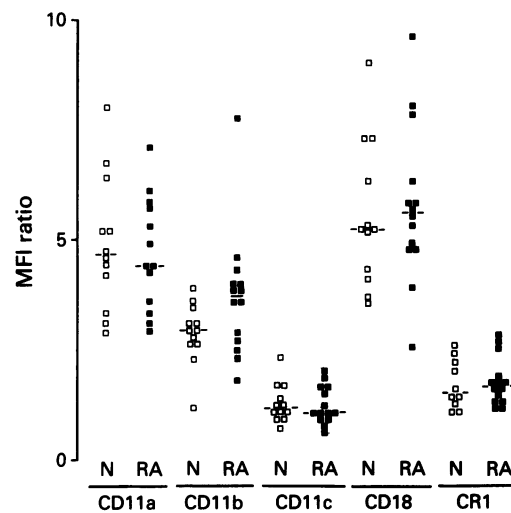

Figure 6   Mean fluorescence intensity ratios of CD11/CD18 and CR1 on polymorphs from control subjects (N) and patients with rheumatoid arthritis (RA). Bars indicate median values.

When neutrophils are activated in vitro the surface expression of CR1 and CR3 (CD11b/CD18) increases in parallel.[30] Possible correlations between the mean fluorescence intensity ratio of CR1 and the mean fluorescence intensity ratios of the components of the CD11/CD18 complex were therefore analysed. The results showed that on control and RA polymorphs, the mean fluorescence intensity ratio of CR1 was correlated only with that of CD18, whereas on control monocytes it was correlated with the mean fluorescence intensity ratios of all components of the CD11/CD18 complex and on RA monocytes with the mean fluorescence intensity ratios of CD11a, CD11b and CD18 (table 4). No significant correlations were found between the mean fluorescence intensity ratios of CR1 and those of the CD11/CD18 complex on lymphocytes from either patients with RA or controls (not shown).

Inflammatory disease activity amongst the patients with RA was correlated with the percentage of polymorphs expressing CD11c ($p<0.0005$), with the percentages of monocytes expressing CR1 ($p<0.05$) and CD11c ($p<0.005$), with the mean fluorescence intensity ratio of CD11c on polymorphs ($p<0.05$) and with the mean fluorescence intensity ratios of CD18 ($p<0.05$), CD11c ($p<0.01$), and CD11b ($p<0.05$) on monocytes (Spearman's rank correlations and two tailed t tests with p values unadjusted for the number of correlations sought). The numbers of circulating lymphocytes, monocytes, and polymorphs in controls and patients with RA were not significantly correlated with either the percentage of cells expressing any of the markers analysed, or the mean fluorescence intensity ratios of those markers.

Discussion

The rapid leucocyte fixation and preparation procedure that was used in these studies was designed specifically to prevent in vitro changes in the surface expression of the leucocyte intergrins. Brief fixation with formaldehyde is known to inhibit the changes in neutrophil

Table 2   Correlations found between the expression of components of the CD11/CD18 complex on different types of leucocytes from patients with RA and controls

α Chain	Leucocytes compared	Control subjects		Patients with RA	
		r	p Value	r	p Value
CD11a	Lymphocytes×polymorphs	0·614	0·034	—	—
CD11b	Monocytes×polymorphs	0·680	0·021	0·609	0·035
CD11c	Monocytes×polymorphs	0·642	0·033	0·607	0·036

Unlisted combinations of leucocytes and those for which values are not indicated (—) had correlation coefficients (r) less than 0·563 and p values greater than 0·05. p Values are unadjusted for the number of comparisons made.

Table 3   Correlations found between the expression of different components of the CD11/CD18 complex on leucocytes from patients with RA and controls

Leucocytes	α Chains compared	Control subjects		Patients with RA	
		r	p Value	r	p Value
Lymphocytes	CD11a×CD11b	—	—	0·780	0·001
Monocytes	CD11a×CD11b	0·819	0·002	0·744	0·006
Monocytes	CD11b×CD11c	0·824	0·002	0·590	0·043

Unlisted combinations of α chains and leucocytes and those for which values are not indicated (—), had correlation coefficients (r) less than 0·552 and p values greater than 0·05. p Values are adjusted for the number of comparisons made.

Table 4   Correlations found between the mean fluorescence intensity ratios of the components of the CD11/18 complex and the mean fluorescence intensity ratio of CR1 on leucocytes from patients with RA and controls

Leucocytes	Markers compared	Controls		Patients with RA	
		r	p Value	r	p Value
Polymorphs	CD18×CR1	0·650	0·042	0·621	0·018
Monocytes	CD11a×CR1	0·899	0·001	0·677	0·016
Monocytes	CD11b×CR1	0·777	0·014	0·686	0·014
Monocytes	CD11c×CR1	0·699	0·036	—	—
Monocytes	CD18×CR1	0·832	0·005	0·828	0·001

Unlisted combinations of markers and leucocytes and those for which no values are indicated (—), all had correlation coefficients (r) less than 0·605 and p values greater than 0·05. p Values are unadjusted for the number of comparisons made.

shape which accompany activation[21] and to preserve the expression of leucocyte surface markers such as CD3, CD4, CD8, and major histocompatibility complex class II (Hamblin et al, unpublished data). Thus, except for any possible changes due to the process of venepuncture itself, we believe that our findings represent the true state of these molecules on the surface of leucocytes from the circulating pool.

Our results show considerable intersubject variation in the expression of CR1 and the components of the CD11/CD18 complex, but are nevertheless broadly consistent with previous findings for normal subjects.[4 5 17 31 32] CD11a/CD18 was expressed on all lymphocytes, monocytes, and polymorphs (figs 1-3), but the level of expression varied from subject to subject (figs 4-6). The percentages of cells expressing CD11b and CD11c varied greatly and the median values were always lower than those for CD11a (figs 1-3). These results are consistent with CD11b and CD11c being weakly expressed on leucocytes.

As yet, the mechanisms which regulate the expression of integrins on leucocytes and which presumably contribute to the substantial intersubject variations in expression are only partly understood. To the best of our knowledge, the correlations noted in tables 2-4 have not previously been reported elsewhere and the biological phenomena responsible for these must be largely a matter for speculation. The fact that similar relative amounts of CD11b and CD11c were expressed on monocytes and polymorphs from the same subject, irrespective of whether they were patients or controls (table 2), is consistent with the expression of these integrins being upregulated on the two cell types in response to activating agents encountered in the circulation. The relative parity in expression of certain combinations of integrins on lymphocytes and monocytes (table 3) may be due to intersubject variations in other mechanisms which regulate coordinately the expression of different integrins—for example, those which control integrin biosynthesis during leucocyte differentiation.

Although the surface expression of CR1 and CR3 (CD11b/CD18) on neutrophils is known to increase in parallel on stimulation,[33] we found no significant correlation between the mean fluorescence intensity ratios of those markers on either control or RA polymorphs. In fact, the mean fluorescence intensity ratio of CR1 seemed better correlated with that of CD18 on polymorphs from the two subject groups (table 4). Interestingly, with the exception of CD11c

on cells from patients with RA, the mean fluorescence intensity ratio of CR1 on monocytes was correlated with each of the components of the CD11/CD18 complex on monocytes from patients with RA and controls (table 4); however, the biological significance of this previously unreported relation is yet to be explained.

Clear but small decreases in the percentage of RA lymphocytes expressing CD11a and CD18 relative to controls were found (fig 1). These results contradict directly an earlier report,[17] in which no difference in the expression of CD11a on RA lymphocytes was found. Larger, but statistically less significant increases, occurred in the percentages of RA monocytes expressing CR1 (fig 2), which, if taken together with similarly significant increases in the mean fluorescence intensity ratios of CR1 and CD11b on these cells (fig 5), is suggestive of previous activation in vivo. The mean fluorescence intensity ratio of CD11b was also increased on monocytes from patients with diabetes, but unfortunately the cells were not also examined for CR1.[34] Our results also differ from those of Heurkens et al[35] who found that RA monocytes expressed significantly less CR1 than control monocytes. Except for a difference in experimental technique (radiolabelling versus fluorescence activated cytometry) there is no obvious explanation for this discrepancy. As increases in the expression of CR1 and/or CD11b have been noted on neutrophils from patients with other inflammatory conditions, such as mild burns,[36 37] haemodialysis induced neutropenia,[38] and systemic lupus erythematosus,[39] it was perhaps surprising that similar increases were not found on RA neutrophils, especially as RA neutrophils show other evidence of activation in vivo[40] and chemotaxin induced quantitative increases in CD11b/CD18 expression precede the qualitative changes that seem to be essential for increased adherence. However, patients with severe burns show a decrease in the percentage of CR1 positive neutrophils and those who develop bacteraemia or pneumonia show a marked reduction in the percentage and absolute numbers of CD11b positive neutrophils.[37] Non-steroidal anti-inflammatory drugs and second line drugs might be expected to affect integrin expression, but no strong conclusions regarding the effects of drugs could be drawn because the number of patients receiving any particular drug or combination of drugs was so small (between one and three). Nonetheless, it is interesting that the only patient receiving no drugs (inflammatory disease activity grade 2) was among the 10 percentile with the lowest mean fluorescence intensity ratios for CD11a,

CD11b, CD11c, and CD18 on lymphocytes and among the 10 percentile with the highest mean fluorescence intensity ratios for CR1 on lymphocytes and for CD11b, CD11c, CD18, and CR1 on monocytes and polymorphs. It is also noteworthy first, that correlations between CR1 and CD11/CD18 expression and disease activity (see text) and between CR3 (CD11b/CD18) expression and neutrophil polarisation[41] all involve cell surface antigens that are known to be upregulated by inflammatory stimuli, and second, that they were observed despite the fact that most patients were receiving anti-inflammatory drugs.

In summary, these studies did not show the marked changes in CR1 or integrin expression on RA polymorphs which might have been expected. However, substantial changes were seen in RA monocytes that were consistent with previous activation in vivo.

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