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Chemotactic factor-induced low density neutrophils express enhanced complement (CR1 and CR3) receptors and increased complement-dependent cytotoxicity

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SUMMARY

We have studied chemotactic factor-induced 'complement receptor enhancement' to determine whether changes in receptor expression and complement-dependent cytotoxicity were associated with alterations in cell density. Ficoll-Paque separated normal human neutrophils (>90%), when further fractionated on discontinuous metrizamide (MTZ) gradients (18, 19, 20, 21, 22, 23% MTZ), consistently gave two major bands at the 20/21% and 21/22% interfaces. Incubation with the synthetic chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP (10^{-8} M)), converted virtually all neutrophils to low density cells sedimenting on MTZ at the 18/19% and 19/20% interfaces. There was a time-dependent change of density after fMLP-stimulation which was maximal at 30 min, with cells reverting towards normal density by 60 min. Control unstimulated cells did not alter their density at any of the time points examined. Activated, low density neutrophils had increased expression of CR1 and CR3 (as shown by flow cytometry and the uptake of ^{125}I-F(ab')$_2$ monoclonal anti-CR1 antibody (E11)). These cells also showed enhanced cytotoxic capacity *in vitro* for helminthic targets (schistosomula of *Schistosoma mansoni*) opsonized with autologous complement. There were highly significant correlations between cell density and anti-CR1 uptake ($P<0.001$), and between schistosomular killing and change in density ($P<0.001$). Increased CR1 expression also correlated with enhanced helminthicidal capacity of neutrophils ($P<0.001$). Complement dependent cytotoxicity was partially reduced after treatment of cells with anti-human CR1 and/or CR3 antibodies, but only in the presence of a second antibody. These findings indicate that chemotactic factor-induced complement receptor enhancement of human neutrophils is associated with a decrease in cell density and increased complement-dependent cytotoxicity (CTX).

Keywords CR1/CR3 cytotoxicity density neutrophil schistosomula

INTRODUCTION

There have been several reports on neutrophil and eosinophil heterogeneity in terms of their density following separation on Percoll or discontinuous metrizamide (MTZ) gradients. Pember *et al.*

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(1983) showed that incubation of murine or human neutrophils with the synthetic chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP), was associated with a decrease in cell density. These low density cells appeared to be 'activated' as shown by partial degranulation and increased myeloperoxidase activity (Pember & Kinkade, 1983). Changes in neutrophil density after exposure to fMLP have also been used as the basis of a rapid method of isolation of normal human blood eosinophils (Roberts & Gallin, 1985).

Similarly, eosinophils from patients with hypereosinophilia separate into low and normal density bands on Percoll or MTZ gradients (De Simone *et al.*, 1982; Winqvist *et al.*, 1982; Prin *et al.*, 1983). Compared to normal density cells the lower density eosinophils appeared to be 'activated', as shown by increased membrane expression for IgG (Fc) (Tai & Spry, 1976; Winqvist *et al.*, 1982) and IgE (Fc) (Capron *et al.*, 1984), enhanced cytotoxicity (Prin *et al.*, 1983), and greater IgG-dependent leukotriene C_4-generating capacity (Shaw *et al.*, 1985).

Enhancement of complement receptors was first reported using the rosette technique (Kay, Glass & Salter, 1979) and later confirmed by flow cytometry (Fearon & Collins, 1983; Berger *et al.*, 1984; Seligmann, Chused & Gallin, 1984). The present study was undertaken to determine whether there was a relationship between neutrophil density and 'complement receptor enhancement (CRE)' following stimulation with chemotactic factors (CF) for either CR1 (Fearon & Collins, 1983; Berger *et al.*, 1984) or CR3 (Berger *et al.*, 1984). In addition, we have measured complement-dependent CTX against a helminthic target *in vitro* to investigate the association between CRE and functional changes in neutrophils.

MATERIALS AND METHODS

Materials are obtained as follows: N-formyl-methionyl-leucyl-phenylalanine (fMLP) and bovine serum albumin (BSA), sodium azide, mouse myeloma proteins, MOPC-21 and UPC-10, (Sigma Chemical Company, Poole, UK), Ficoll-Paque (Pharmacia, Milton Keynes, UK), Hanks BSS and RPMI 1640 (Gibco, Paisley, Scotland, UK), dibutyl phthalate and dinonyl phthalate (BDH Chemicals, Poole, UK), Dextran 110 (Fisons, Loughborough, UK) and metrizamide (MTZ) (Nyegaard Ltd, Birmingham, UK). Anti-CR1 and anti-CR3 (Leu 15) monoclonal antibodies (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA, USA). Fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit antibody to mouse immunoglobulin (Dako Ltd, High Wycombe, Bucks, UK).

Neutrophil isolation and separation. Peripheral blood obtained from healthy donors was added to 3·8% trisodium citrate and Dextran (9:1:2 ratio). Leucocyte-rich plasma was layered over Ficoll-Paque at 4°C and centrifuged at 4°C for 20 min at 400 ***g***. The granulocyte pellet was resuspended in cold lysis buffer (ammonium chloride 8·2 g and $KHCO_3$ 1·0 g in 1 litre, pH adjusted to 7·2–7·4) and centrifuged at 4°C for 7 min at 200 ***g***, washed twice in cold Hank's balanced salt solution. The leucocytes were pooled, counted and suspended in cold Hank's BSS to a concentration of 1 to 2×10^7 cells/ml, and incubated (37°C for 30 min) with fMLP or Hank's. Aliquots were taken from each, adjusted to 5×10^6 cells/ml and kept on ice for comparison with MTZ separated cells. fMLP-stimulated and control cells were kept on ice for 30 min, then centrifuged at 200 ***g*** at 4°C for 7 min, resuspended to 2 ml, and applied to the MTZ gradient (Vadas *et al.*, 1979) with the following modifications. MTZ (w/v%) gradient was layered from 23% concentration (1·1232 g/ml density) up to 18% (1·0957 g/ml), rising 1% at each step (see Fig. 1). MTZ gradients were centrifuged at 1200 ***g*** for 45 min at 20°C and the neutrophil bands were removed, washed twice in RPMI 1640 and counted (both total and differential). The cells were reconstituted to 5×10^6 cells/ml and only fractions with >90% neutrophils were used in the assays described below.

Radioligand binding assay. An anti-CR1 monoclonal antibody (E11) was used as previously described (Hogg *et al.*, 1984; Richerson *et al.*, 1985). Briefly, $2{\cdot}5 \times 10^6$ neutrophils were incubated at 4°C for 45 min with 0·25 μg ^{125}I-E11 in a total volume of 750 μl of Hanks BSA containing 0·04% sodium azide. Triplicate 150 μl samples of the mixtures were then layered over 200 μl of dibutyl phthalate and dinonyl phthalate (4:1 ratio) in 400 μl polypropylene microfuge tubes. After the

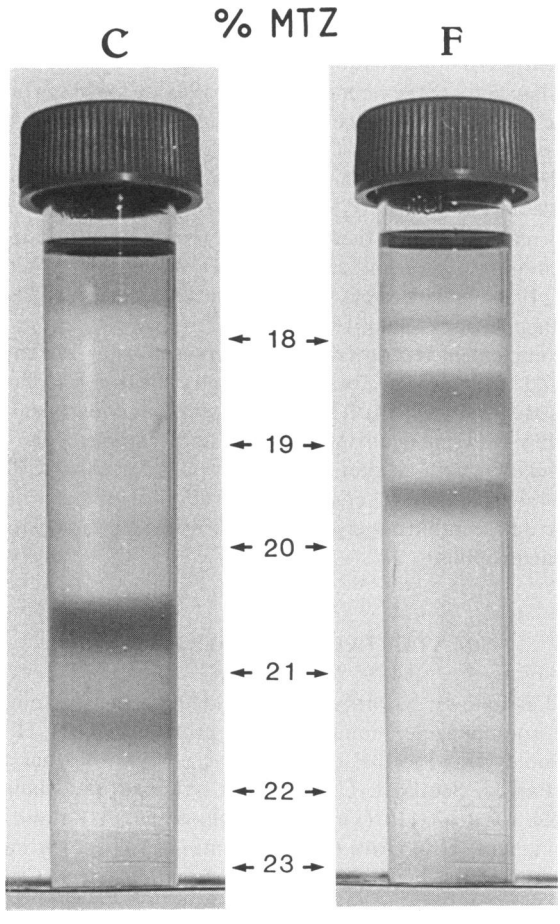

Fig. 1. Changes in density of normal (C) and fMLP-stimulated (F) human neutrophils observed on a discontinuous metrizamide MTZ gradient. The various density percentages of MTZ are arrowed (at the middle of each concentration). Density values of each interface from 18% downwards were as follows: 1·0957, 1·1012, 1·1067, 1·1122, 1·1177 and 1·1232 g/ml. Neutrophils were concentrated on a Ficoll-Paque gradient, incubated with either buffer (C) or fMLP 10^{-8} M (F) for 30 min at 37°C before MTZ separation. Neutrophils sedimented in two bands (at 20/21% and 21/22% interfaces (1·1122 and 1·1177 g/ml, respectively) for control cells and at 18/19% and 19/20% interfaces (1·1012 and 1·1067 g/ml, respectively) for stimulated cells).

tubes were centrifuged at 800 ***g*** for 2 min, they were frozen in a methanol/dry-ice bath, the tips containing the cell pellets were collected and the cell-bound radioactivity measured in a gamma counter. The number of antibody molecules per cell was calculated using the following equation $(a-b/c) \times 1{\cdot}2 \times 10^6$ where a = triplicate sample mean, b = background mean, and c = counts of a standard containing 100 ng ^{125}I-E11. It was assumed that 1 mole of antibody contains $6{\cdot}024 \times 10^{23}$ molecules; thus, 100 ng contains 6×10^{11} molecules which, when divided by the number of cells present (5×10^5), gives $1{\cdot}2 \times 10^6$.

Non-specific binding of labelled E11 was calculated by saturating the cells with 'cold' unlabelled E11 (4°C, 45 min) followed by incubation with ^{125}I labelled E11. This resulted in less than 1% binding of the label. Approximately 10-fold molar excess of unlabelled E11 was used to saturate the receptors on the cells before adding the ^{125}E11 (Walport *et al.*, 1985).

Immunofluorescence. Samples of 5×10^5 neutrophils were resuspended in 50 μl of ice-cold phosphate buffered saline (PBS) containing 0·1% NaN_3 and 0·5% BSA (PAB) and incubated with saturating amounts of anti-CR1 or anti-CR3 monoclonal antibodies for 15 min at 4°C. After

Table 1. Change in the number of ^{125}I-E11 molecules bound per cell and percentage cytotoxicity (ability of cells to kill C-coated schistosomula of *S. mansoni*) before (control) and after fMLP (10^{-8} M)-stimulation (30 min at 37°C)

	CR1 ($\times 10^3$)		CTX (% Kill)	
Expt No	Control	fMLP	Control	fMLP
1	6·5	77·7	32·0	56·0
2	17·4	77·3	30·0	55·0
3	8·4	26·7	19·0	35·6
4	16·0	37·5	21·0	34·5
5	7·4	22·5	22·5	36·0
6	28·5	78·0	29·0	50·0
7	14·1	28·2	25·5	37·0
8	9·9	21·9	28·5	50·0
Mean (± s.e.m.)	13·5 ± 2·6*	46·5 ± 9·3†	26·0 ± 1·6*	44·3 ± 3·3†

The neutrophil-rich cell preparations were obtained after Ficoll-Paque separation of dextran-sedimented human leucocytes. Cells kept at 4°C throughout these manipulations had a mean CR1 count of $5{\cdot}3 \pm 1{\cdot}8 \times 10^3$.

*† Using a Mann–Whitney *U*-test the statistical significance of the difference between these values in both CR1 and CTX was $P < 0{\cdot}001$.

washing once with 4 ml of cold PAB, FITC-conjugated F(ab′)$_2$ fragments of rabbit antibody to mouse immunoglobulins was added in excess and the cells incubated for a further 15 min at 4°C. After washing, the cells were resuspended in PBS containing 0·1% NaN_3 and kept on ice until analysed. Non-specific binding of anti-CR1 and anti-CR3 antibodies was determined by incubating cells with identical concentrations of mouse myeloma proteins of the same antibody isotype (MOPC-21 for anti-CR1 and UPC-10 for anti-CR3) but with irrelevant antigen specificity. Fluorescence intensity was determined on 10,000 cells from each sample by a Becton-Dickinson FACS Analyzer, standardized with fluorescent microbeads, using logarithmic amplification which was converted to the linear equivalent by a Hewlett-Packard Consort 30 Computer. Samples were compared by their arithmetic means and specific fluorescence attributable to CR1 or CR3 was calculated by subtracting the mean fluorescence of cells incubated with control antibodies from that of cells stained with specific monoclonal antibodies. The results from replicate experiments are expressed as the mean ± 1 s.e.m.

Cytotoxicity. Details of the schistosomula killing assay have been described elsewhere (Anwar, Smithers & Kay, 1979; Moqbel *et al.*, 1983). The total reaction volume was 150 μl, which contained 50 μl of schistosomula (50 larvae), 50 μl of autologous fresh serum (complement (C) final dilution of 1:3) and 50 μl of neutrophils (at a concentration of 4×10^6/ml) obtained from activated (fMLP-treated) or control cells suspensions before or after MTZ gradient separation. The final cell: schistosomula ratio was 4000:1. The assay which also included other relevant controls (as previously described (Anwar *et al.*, 1979; Moqbel *et al.*, 1983) was assessed 20 h later and percentage of dead schistosomula (i.e. CTX) was determined in a visual assay.

RESULTS

Ficoll-Paque enriched neutrophils from six normal, healthy donors were incubated for 30 min, in the presence or absence of fMLP (10^{-8} moles/l), and the changes in ^{125}I-E11 uptake (CR1 expression) were compared with the percentage CTX (Table 1). CR1 expression and CTX were significantly enhanced ($P < 0{\cdot}001$) in fMLP-treated cells compared to normal unstimulated neutrophils. The cells were then layered on MTZ and the positions of neutrophil bands following

Table 2. The number of ^{125}I-E11 molecules bound per cell (CR1) and percentage cytotoxicity (CTX%) against C-coated schistosomula in neutrophil-rich (>90%) bands sedimenting at various interfaces of a discontinuous MTZ gradient in the presence or absence of 10^{-8} M fMLP

Experiment	Stimulus	Interface (% MTZ)	Density (g/ml)	Neutrophils (% of total WBC)	CR1		CTX	
					(×10^3)	(% increase)	(% kill)	(% increase)
1	Control	20/21	1·1122	96	44·2	0	32	0
	fMLP	18/19	1·1012	97	57·8	(30%)	48	(50%)
	fMLP	D/18*	1·0957	96	96·9	(119%)	49	(53%)
2	Control	19/20	1·1067	92	51·0	0	32	0
	Control	20/21	1·1122	94	40·5	0	38	(18%)
	fMLP	18/19	1·1012	96	56·3	(37%)	46	(43%)
3	Control	20/21	1·1122	97	23·9	0	17	0
	Control	19/20	1·1067	100	29·4	0	20	0
	fMLP	19/20	1·1067	99	54·6	(129%)	26	(53%)
	fMLP	18/19	1·1012	94	57·0	(139%)	37	(117%)
4	Control	20/21	1·1122	97	33·3	0	18	0
	Control	19/20	1·1067	95	38·1	0	19	0
	fMLP	19/20	1·1067	97	62·0	(79%)	28	(55%)
	fMLP	18/19	1·1012	90	56·7	(64%)	40	(144%)
5	Control	20/21	1·1122	100	37·2	0	20	0
	Control	21/22	1·1177	100	35·9	0	21	0
	fMLP	19/20	1·1067	99	70·2	(88%)	34	(70%)
6	Control	20/21	1·1122	98	26·7	0	22	0
	Control	19/20	1·1067	95	27·8	0	26	0
	fMLP	18/19	1·1012	96	60·3	(117%)	43	(95%)
7	Control	20/21	1·1122	97	30·5	0	31	0
	fMLP	19/20	1·1067	96	83·7	(175%)	50	(61%)
	fMLP	18/19	1·1012	99	55·0	(80%)	47	(51%)
8	Control	20/21	1·1122	100	18·9	0	21	0
	fMLP	19/20	1·1067	94	47·6	(151%)	58	(176%)

Figures in parentheses represent % increase in values, when compared with baseline control values, in each experiment. D/18* indicates that cells were found above the 18% MTZ interface. Cells were incubated with fMLP (10^{-8} M) for 30 min at 37°C before loading on MTZ gradients.

centrifugation are shown in Fig. 1. With untreated cells, two major neutrophil bands were consistently observed at the 20/21% and 21/22% interfaces (1·1122 and 1·1177 g/ml, respectively). Following treatment with fMLP most of the neutrophils were observed at 18/19% and 19/20% interfaces (1·1012 and 1·1067 g/ml, respectively). Contaminating eosinophils, when present, gave a faint band at the 22/23% interface (1·1232 g/ml) but were invariably found at the 21/22% interface (1·1177 g/ml) after fMLP stimulation (data not shown). The less defined bands with lowest density fractions (18/19%, 1·1012 g/ml) contained contaminating mononuclear cells. Following fMLP treatment, a small number of low density neutrophils were found to contaminate the mononuclear interface (above 18%, 1·0957 g/ml). There was a degree of variation in the purity of neutrophils (ranging from 68–98%) in the various bands shown in Fig. 1. Contaminating cells were either eosinophils or mononuclear cells. Only those MTZ fractions which contained 90% or more neutrophils were used for complement receptor expression and CTX studies (Table 2). The fMLP-induced low density neutrophils had enhanced CR1 expression (^{125}I-E11 uptake) and demonstrated increased capacity to kill schistosomula compared with unstimulated control cells (C) (Table 2).

The time course of change in CR1 and CTX of fMLP-stimulated Ficoll-Paque enriched neutrophils (before MTZ separation) is shown in Table 3. There was 3–4-fold increase in mean ^{125}I-E11 uptake of stimulated cells at 30 min and these differences were maintained at the 60 min time point. Although there were increases in complement-dependent CTX, the differences between

Table 3. Mean ± s.e.m. ($n = 3$) of a time course study of change in ^{125}I-E11 uptake and percentage cytotoxicity against schistosomula of human neutrophils concentrated on Ficoll-Paque gradient and incubated for 15, 30 and 60 min with either fMLP (at 10^{-8} M concentration) or control buffer, before loading on MTZ gradients

	Time of incubation (min) with fMLP (10^{-8} M)					
	15		30		60	
	Control	fMLP	Control	fMLP	Control	fMLP
CR1 ($\times 10^3$)	15·0* (±5·1)	30·0† (±15·0)	17·4* (±6·0)	42·8† (±18·0)	19·8* (±6·6)	43·5† (±7·5)
CTX (% kill)	26·3‡ (±6·4)	31·2§ (±2·2)	27·6* (±1·0)	45·6† (±4·3)	29·0* (±1·7)	39·0† (±3·0)

The differences between the mean values were as follows: * vs † $P < 0{\cdot}01$; ‡ vs § NS (Mann–Whitney U-test).

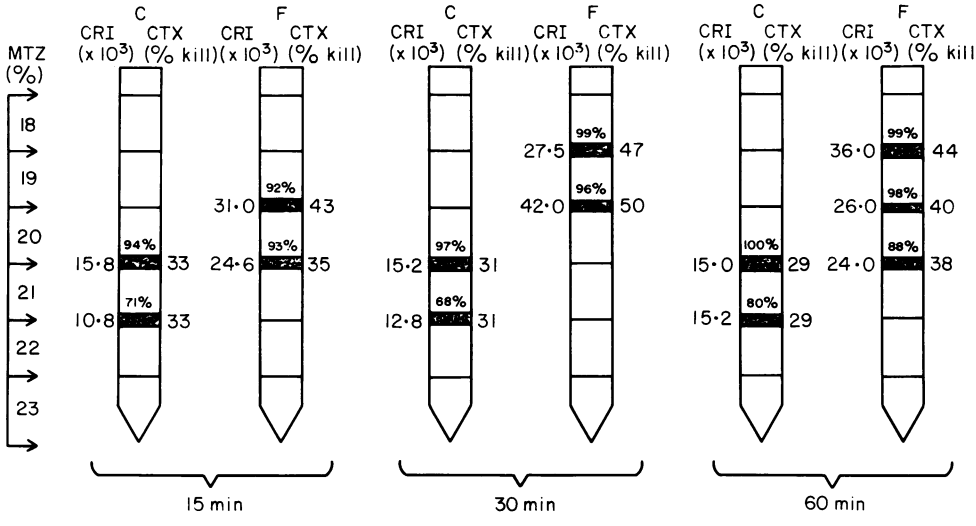

Fig. 2. A diagrammatic representation of a time course study showing changes in density and function of neutrophils following treatment with either fMLP (F) or control buffer (C). Numbers over the band represent the percentage of neutrophils of the total cell count in each band. CR1 = number of ^{125}I-E11 molecules bound per cell. CTX (%) = percentage cytotoxicity (killing of schistosomula). The experiment was performed on two further occasions and gave essentially similar results. For MTZ density values (g/ml) refer to Fig. 1.

stimulated and control cells were less marked than for CR1. However there were clear differences in CTX at 30 and 60 min time points between fMLP-treated cells and controls.

Following MTZ fractionation, lower density neutrophils were associated with a time-dependent increase in the uptake of ^{125}I-E11 and CTX; a representative experiment is shown in Fig. 2. At 15 min, stimulated neutrophil bands had moved a single interface upwards, whereas at 30 min there was a two-interface shift (as in Fig. 1). At 60 min, there was a tendency to revert to the 15 min pattern. This experiment was repeated three times and yielded similar results. In the latter experiments, careful precautions were taken to ensure that our physiological buffers and Ficoll-Paque gradient were endotoxin-free (*Limulus* test-negative, LAL-GEL Kit, Laboratory Impex Ltd,

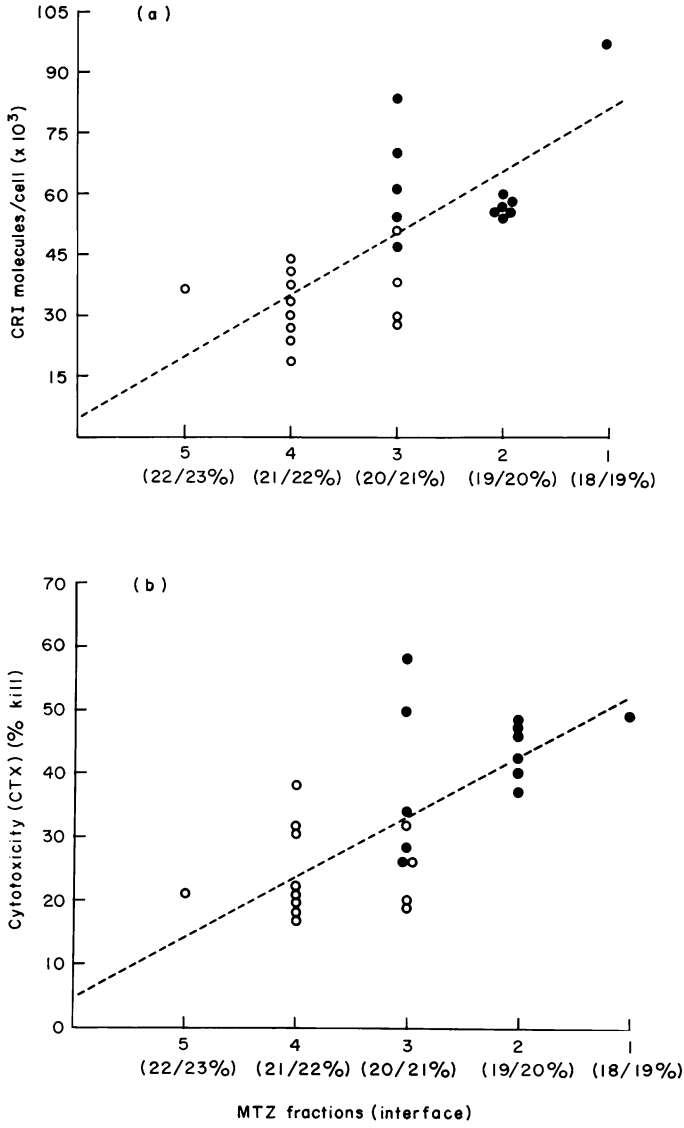

Fig. 3. Correlation between: (a) complement receptor expression (measured by the numbers of ^{125}I-E11 molecules bound per cell) and density of neutrophils (expressed by the interface they occupy on a discontinuous MTZ gradient); and (b) neutrophil cytotoxicity (measured by enhanced killing of C-coated schistosomula of *S. mansoni*) and cell density (% MTZ). Neutrophils were incubated with either fMLP (●) or buffer (○). Schistosomula killing was measured at 20 h. For MTZ density values (g/ml) refer to Fig. 1. (a) $r = -0{\cdot}708$, $P < 0{\cdot}001$; (b) $r = -0{\cdot}667$, $P < 0{\cdot}001$.

Twickenham, UK). These experiments gave identical results, particularly in regard to the two bands of neutrophils (pre- and post-activation) observed on MTZ gradients. This was further confirmed when we omitted the Ficoll-Paque step before layering the cells on the MTZ gradient.

There was a highly significant ($P < 0{\cdot}001$) inverse correlation between the uptake of ^{125}I-E11 and cell density (Fig. 3a) and between the functional capacity of neutrophils (CTX) and changes in cell density (Fig. 3b). In addition, a highly significant positive correlation was observed between CR1 expression and enhanced complement-dependent CTX ($n = 25$, $r = 0{\cdot}703$, $P < 0{\cdot}001$, data not shown). In order to confirm this observation, we have attempted to block CR1/CR3 dependent

adherence and CTX by treatment of neutrophils with a saturating dose of anti-CR1, anti-CR3 or a mixture of both in the presence or absence of a second, rabbit anti-mouse, antibody ($n=3$). Direct blocking of C-dependent CTX was not achieved with anti-CR1, anti-CR3 or both. Such treated cells showed a relatively small (19–29%) increase in killing when compared with their baseline controls which included cells treated with irrelevant control immunoglobulins of similar isotypes (i.e. IgG_1 and IgG_{2a} respectively). Partial inhibition (48%) of CTX was observed but only in the presence of the second antibody. When treated cells (at 4°C) were incubated at 37°C, both capping and internalization of receptors were observed microscopically and this was dependent upon the incubation time.

Indirect immunofluorescent staining of neutrophil complement receptors gave results similar to ^{125}I-E11 uptake for CR1. Pre-incubation of cells with fMLP resulted in a 4-fold increase in specific mean fluorescence of CR1 and greater than 5-fold enhancement of CR3 compared with 37°C control, illustrating that changes in CR1 and CR3 expression are similar in this system (Fig. 4). A temperature-induced change in expression can also be seen for both receptor types, as well as a small effect of purification procedures.

DISCUSSION

Several investigators have pursued the observation made by Kay *et al.* (1979) showing that *in vitro* chemotactic factor-induced cell activation is associated with enhanced complement receptor expression. This phenomenon was further confirmed by flow cytometry for both CR1 and CR3 (Fearon & Collins, 1983; Berger *et al.*, 1984; 1985).

Cell activation by chemoattractants is a complex process involving a number of cyto-physiological alterations which include, amongst others, changes in density and volume (Pember *et al.*, 1983). The results of our study have demonstrated a link between the above two phenomena with increases in the functional capacity of human neutrophils following *in vitro* stimulation.

Human neutrophils (separated by Ficoll-Paque) and activated by fMLP showed increased uptake of ^{125}I-E11, as previously shown by Richerson *et al.* (1985), but in addition demonstrated an enhanced capacity to kill complement-coated helminthic targets (Table 1). This suggested a possible association between complement receptor enhancement and complement-dependent CTX. After further separation of cells on MTZ it was evident that activation was associated with decreased density (Fig. 1 and Table 2).

The MTZ densities used (18, 19, 20, 21, 22, 23%) were different from those employed by Vadas *et al.* (1979) (18, 20, 22, 23, 24, 25%) for separating eosinophils. In fact, the changes observed in neutrophil density could only be appreciated with the 1% increments in MTZ used in the present studies. Before activation all of the normal neutrophils studied gave two bands on MTZ (Fig. 1). This was usually at the 20/21% and 21/22% interfaces (1·1122 and 1·1177 g/ml, respectively), although occasionally normal cells sedimented at the 20/21% and 19/20% (1·1067 and 1·1122 g/ml, respectively). In Table 2 we presented data from eight individual experiments, which included only those bands containing 90% or more neutrophils. Some variability was observed in baseline values of CR1 between individuals before fMLP simulation (Table 1). This may be due to varying degrees of activation of the cells obtained from three different donors in these experiments. Similarly, an apparent temporary dissociation may be seen between upregulation of CR1 and enhanced CTX (Table 3) although a trend in change with both events was evident. It is possible that enhancement in CTX requires a particular threshold level of enhancement in CR1 values before it is reflected in increased larval killing. Anti-CR1 binding may not necessarily be related to the functional expression of this receptor (Richerson *et al.*, 1985).

It was previously established that complement receptor enhancement (as assessed by anti-CR1) occurs when samples are simply warmed from 4°C to 37°C in the absence of chemotactic factor and that further enhancement by fMLP was minimal (Fearon & Collins, 1983). Subsequent studies demonstrated that fMLP-stimulation was significantly above that observed when following incubation at 37°C (Berger *et al.*, 1984; 1985). Richerson *et al.* (1985) described the effect of a number of variables on anti-CR1 uptake by neutrophils. This included the effect of initial dextran

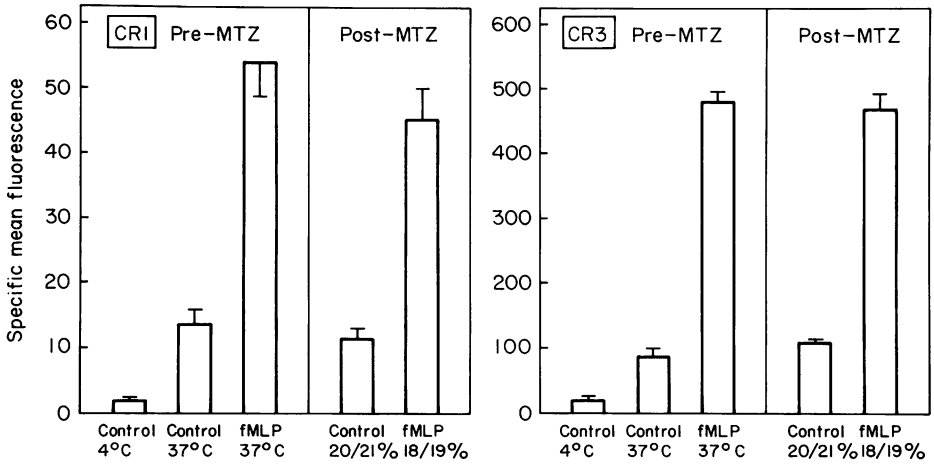

Fig. 4. CR1 and CR3 expression on human neutrophils measured by flow cytometry. Specific mean fluorescence values of cells separated on Ficoll-Paque (Pre-MTZ, $n=3$) and stimulated with fMLP (10^{-8}, 37°C) or control diluent at both 4°C and 37°C are compared with values of cells obtained from discontinuous MTZ gradient after similar treatment ($n=5$). Post-MTZ cells were obtained from the major band of the gradient, the 20/21% interface (1·1122 g/ml density) for the control cells and the 18/19% interface (1·1012 g/ml) for fMLP-treated cells.

sedimentation at 4°C, 20°C and 37°C, as well as Ficoll-Paque at various conditions, and concluded that no major changes were observed in the stability of CR1. In the present studies, all cell manipulations following dextran sedimentation and before activation with fMLP were undertaken at 4°C, and the cells were left on ice for 60 min after fMLP-stimulation before layering on the MTZ gradients in order to reduce heat-induced change in density and CR1 profile. We have observed (R. Moqbel, H. Richerson, A. J. MacDonald, unpublished data) that warming cells to 37°C immediately before gradient separation was in fact associated with a one interface change in density.

In this study, we have used the method of radioligand binding of ^{125}I-F(ab′)$_2$ anti-CR1 in order to quantify precisely the number of CR1 molecules per cell. In addition, we have used flow cytometry to confirm this observation and extend it to compare the upregulation of CR3 with CR1 following stimulation. The percentage enhancement of CR3, as assessed by increased specific fluorescence, was comparable to that observed with CR1, although the intensity of fluorescence of CR3 was stronger (Fig. 4). The complement-dependent schistosomula killing assay is a well-established test of granulocyte function and is known to be mediated through C3b (Anwar *et al.*, 1979). Whether enhancement of C-dependent killing by activated, low density neutrophils is dependent on an increase in CR1 and CR3 remains speculative. Our attempt to inhibit killing of C-coated schistosomula using a mixture of mouse anti-CR1 and anti-CR3 only achieved partial inhibition when a second (rabbit anti-mouse) antibody was added to increase the steric hindrance and physically separate the target from the opsonically active receptor. Anti-CR1 and anti-CR3 monoclonal antibodies, when added singly or mixed together, did not result in inhibition, suggesting that these antibodies may bind to a site on the receptor not involved in the expression of its adherence function.

The alterations in cell density induced by fMLP may be the result of a number of factors. It is known that activation of neutrophils by chemotactic agents results in membrane ruffling and shape changes (Yuli & Snyderman, 1984; Sklar, Oades & Finney, 1984), as well as increased adherence (Craddock *et al.*, 1977; Gallin, Durocher & Kaplan, 1985). Pember *et al.* (1983) showed that the decrease in cell density was associated with increased cell size which was also observed in our FACS analysis (A. Hartnell & R. Moqbel, unpublished data).

The bands observed at the 20/21% and 21/22% MTZ interfaces in unstimulated neutrophils might be a further example of neutrophil heterogeneity (reviewed by Gallin & Seligmann, 1984). This possibility is currently being assessed by comparing the two fractions using a number of tests of leucocyte function.

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