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Expression of lactoferrin on human granulocytes: analysis with polyclonal and monoclonal antibodies

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SUMMARY

Lactoferrin (LF), an iron-binding protein present in specific granules of neutrophils, is expressed on membrane after granulocyte activation. It may represent a target for anti-neutrophil cytoplasmic antibodies (ANCA) in patients affected by some immunomediated diseases. We recently produced two MoAbs, AGM 2.29 and AGM 10.14, that recognize two spatially distant epitopes of human LF. In this study we perform a cytometric analysis in order to evaluate the expression of LF on the surface of granulocytes obtained from freshly drawn blood or after purification, in both the presence and absence of stimuli. Our results demonstrate that LF is not constitutively expressed on membrane of circulating neutrophils. After priming with phorbol myristate acetate (PMA) or tumour necrosis factor-alpha (TNF-α), an increased mean fluorescence intensity (MFI) was obtained on neutrophils stained with polyclonal anti-LF antibodies and with AGM 2.29. The kinetics of LF expression during activation demonstrated a progressive increase in MFI within 45 min. No increase in MFI was documented when primed granulocytes were stained with MoAb AGM 10.14, thus indicating that the epitope recognized by AGM 10.14 is not exposed at the cell surface. Following membrane permeabilization, performed in order to analyse the binding of anti-LF MoAbs to cytoplasmic LF, a marked increase in MFI was obtained by staining granulocytes with both anti-LF MoAbs. Indirect immunofluorescence (IIF) analysis confirmed that AGM 2.29 and AGM 10.14 reacted with human granulocytes, showing a cytoplasmic pattern on formalin–acetone-fixed neutrophils and a perinuclear one on ethanol-fixed cells.

Keywords lactoferrin granulocytes cytometric analysis monoclonal and polyclonal antilactoferrin antibodies

INTRODUCTION

Lactoferrin (LF) is an iron-binding protein, present not only in the cytoplasmic granules of neutrophils but also in milk, tears and secretions. It exerts an antibacterial effect by depriving bacteria of iron and by damaging the outer membrane of Gram-negative microbes [1]. LF also prevents the formation of hydroxyl radicals [2], as well as inhibiting complement activation, through the inhibition of C3 convertase [3]. More recently, it has been suggested that LF may act as a 'multifunctional immunoregulatory protein' [4].

On the other hand, LF represents a target for anti-neutrophil cytoplasmic antibodies (ANCA). It is well known that other targets of this family of autoantibodies are proteinase-3 (PR3) and myeloperoxidase (MPO): anti-PR3 antibodies are strongly associated with Wegener's granulomatosis [5], anti-MPO antibodies have been reported in crescentic glomerulonephritis and other forms of vasculitides [6–8]. Anti-LF antibodies have been detected in different forms of chronic inflammatory disorders, such as rheumatoid arthritis (RA) [9,10] and inflammatory bowel disease (IBD) [11]. Falk *et al.* demonstrated that ANCA directed against PR3 or MPO induce *in vitro* the respiratory burst and degranulation of normal donor granulocytes primed with tumour necrosis factor-alpha (TNF-α) [12]. Recently Mulder *et al.* documented that LF also becomes accessible at the cell surface after priming of granulocytes: this process is FcRII-dependent and results in induction of the respiratory burst [13].

Most studies on LF expression and effects of anti-LF antibodies have been performed utilizing polyclonal antibodies. We recently produced two new MoAbs recognizing spatially distant epitopes of human LF that react with ethanol-fixed granulocytes showing a perinuclear staining pattern [14].

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The objective of this study was to analyse the constitutive expression of LF on surface membrane of circulating granulocytes, either from freshly drawn blood or obtained after purification, both in the presence and in the absence of stimuli.

MATERIALS AND METHODS

Reagents

Dextran 60·000, phorbol myristate acetate (PMA), caprylic acid, ammonium sulfate, human LF (purified from human milk), bovine serum albumin (BSA), Freund's incomplete adjuvant (FIA), FITC-conjugate $F(ab')_2$ fragments of sheep antibodies to mouse IgG and pristane were purchased from Sigma Chemical Co. (St Louis, MO). PMA was dissolved in DMSO and stored in sterile containers at −80°C. Lymphoprep was obtained from Nycomed Pharma AS (Oslo, Norway). FACS lysing solution ×10 concentrate (a buffered solution containing < 15% formaldehyde and < 50% diethylene glycol) was purchased from Becton Dickinson (San Jose, CA).

Recombinant TNF-α (a gift from Pharmacia-Upjohn, Milan, Italy) was stored at −80°C in small aliquots until use.

All reagents utilized throughout the experiments were uncontaminated by bacterial lipopolysaccharide (LPS), when tested using the Limulus lysate assay (Sigma).

Preparation of anti-LF immune antiserum

Four 8-week-old female BALB/c mice were primed with an intraperitoneal (i.p.) injection of 100 μg LF mixed with FIA. On days 14 and 28 the mice were given an i.p. injection of 100 μg of the same immunogen. On day 35 blood was taken and sera pooled. The specificity of this antiserum was evaluated by means of an ELISA.

Anti-LF antiserum and control normal mouse immunoglobulin were inactivated at 56°C for 30 min before experiments.

Monoclonal antibodies (MoAbs)

Ascites was produced by injecting 2×10^6 anti-LF MoAb-producing cells into pristane-primed BALB/c mice. MoAbs (named AGM 10.14, an IgG1, and AGM 2.29, an IgG2b) were purified from ascitic fluid by sequential precipitation with caprylic acid and 45% ammonium sulfate [15,16]. The purity of the MoAbs was assessed by SDS–PAGE [17]. The characterization of AGM 2.29 and AGM 10.14 has been described elsewhere [14].

Evaluation of the constitutive expression of LF on granulocytes

To evaluate the expression of LF on the surface membrane of circulating neutrophils, 100 μl of freshly drawn blood were immediately mixed with 400 μl of ice-cold PBS containing 0·1% NaN_3. After centrifugation, the pellet was incubated with polyclonal anti-LF antibodies or MoAbs AGM 10.14 and AGM 2.29, and treated as described below ('flow cytometry studies').

Purification of granulocytes

Heparinized blood from healthy subjects was collected in sterile tubes and purified by sequential dextran (6% w/v in saline) sedimentation, Lymphoprep density gradient centrifugation (30 min at 400 ***g***). The pellet, containing both erythrocytes and granulocytes, was treated for 1 min with hypotonic solution. Cells were then washed twice in ice-cold PBS. Cell viability, assessed by trypan blue exclusion, was > 95% at the beginning and end of all experiments.

Priming of granulocytes

Priming of purified granulocytes as well as granulocytes of freshly drawn blood was performed at 37°C by incubating the cells for 5, 15, 30 and 45 min with either PMA (1 μg/ml in PBS) or TNF-α (2 ng/ml in PBS). Following incubation the reaction was stopped by adding 1 ml of ice-cold PBS containing 0·1% (w/v) NaN_3. Then the cells were centrifuged at 4°C for 10 min.

Flow cytometry studies

After washing, either primed or non-primed cells (5×10^5/ml) were incubated with 20 μl of 1:200 dilution of anti-LF antiserum or with 20 μl of purified anti-LF MoAbs at a concentration of 50 μg/ml. Both serum and MoAbs were diluted in ice-cold PBS/0·1% NaN_3. After a 30-min incubation period at 4°C, cells were washed in PBS/0·1% NaN_3 and incubated with an appropriate dilution of FITC conjugate $F(ab')_2$ fragments of sheep antibodies to mouse IgG. Controls were performed either without a primary antibody or by incubating cells with 20 μl of 1:200 dilution of normal BALB/c mouse serum. In the experiments performed on freshly drawn blood, erythrocytes were lysed by Becton Dickinson lysis solution at a dilution of 1:10, following the manufacturer's instructions. Cytometric analysis of surface antigen expression was performed on a Becton Dickinson FACScan using Lysis II software. When leucocytes from freshly drawn blood were analysed, cells other than granulocytes were gated away by forward and side light scatter. Ten thousand cells were counted in each analysis. Results are given as mean fluorescence intensity (MFI).

Inhibition of anti-LF serum and anti-LF MoAb binding to granulocytes by soluble LF

Mouse anti-LF antiserum (final dilution 1:200) and anti-LF MoAbs (final concentration 20 μg/ml) were preincubated with molar excess of soluble LF (final concentration 500 μg/ml), for 1 h at room temperature. Then, 20 μl of the mixture were incubated with granulocytes and flow cytometry study was continued as described above. As negative controls anti-LF antiserum and anti-LF MoAbs were preincubated with BSA at the same concentration of soluble LF.

Binding of anti-LF MoAb to cytoplasmic LF

The binding of MoAb to cytoplasmic LF was determined following permeabilization of granulocytes. In brief, 100 μl of freshly drawn blood were preliminarily incubated with 2 ml of 1:10 dilution of a FACS lysing solution, as cell membrane permeabilizing agent, at room temperature for 10 min and then centrifuged at 300 ***g*** for 5 min. Cells were washed twice in PBS at 300 ***g*** for 5 min and then incubated for 30 min with AGM 2.29 and AGM 10.14, respectively. After two washings in PBS, the cells were incubated with an appropriate dilution of FITC conjugate $F(ab')_2$ fragments of sheep antibodies to mouse IgG. Controls were performed without a primary antibody or by incubating cells with 20 μl of 1:200 dilution of normal BALB/c mouse serum. Finally, cells were washed in PBS/1% Tween-20 at 300 ***g*** for 5 min, in order to minimize the aspecific fluorescence, and resuspended in PBS/0·1% NaN_3. Cytometric analysis was performed as previously described.

Indirect immunofluorescence on human granulocytes

Purified granulocytes from a healthy donor were cytocentrifuged on glass slides and fixed either in 99% ethanol for 5 min at 4°C [18] or in formalin-acetone according to the method of Pryzwansky *et al.* with slight modifications (0·5% formalin, 45% acetone in

PBS, pH 7·4, at 4°C for 45 s without washing after fixation) [19,20]. Polyclonal antiserum at a dilution of 1:50, and MoAbs (20 μg/ml) were then placed in a moist chamber for 30 min. Following three washings with PBS, and incubation for another 30 min with FITC-conjugated $F(ab')_2$ fragments of sheep anti-mouse IgG, the slides were again washed with PBS, mounted with PBS-glycerol, and observed under a fluorescence microscope (Leitz Orthoplan, Wetzlar, Germany).

RESULTS

Expression of LF on freshly drawn neutrophils

To evaluate the constitutive expression of LF on surface membrane of circulating neutrophils, freshly drawn blood was mixed with ice-cold PBS/0·1% NaN_3 immediately after venepuncture. No LF expression was demonstrated either by incubating neutrophils with polyclonal anti-LF antibodies or MoAbs AGM 10.14 and AGM 2.29.

When neutrophils were primed with PMA or TNF-α, binding of polyclonal anti-LF and AGM 2.29 resulted in an increase in MFI when compared with normal mouse IgG. In the representative case illustrated in Fig. 1, increases in MFI of 240% and 214% were found on PMA-primed neutrophils stained with polyclonal anti-LF and MoAb AGM 2.29, respectively. By contrast, no increase in MFI was documented when primed granulocytes were stained with MoAb AGM 10.14 (not shown).

The kinetics of LF expression on PMA- or TNF-α-primed neutrophils from five normal subjects is shown in Fig. 2. Following stimuli, an increase in MFI was found, both by staining neutrophils with polyclonal anti-LF and, to a lesser extent, AGM 2.29. MFI progressively increased on PMA- or TNF-α-primed granulocytes within 45 min.

Inhibition of polyclonal and monoclonal anti-LF antibody binding to granulocytes by soluble LF

The specificity of polyclonal and monoclonal anti-LF antibody binding was analysed by means of inhibition experiments with soluble LF. Preincubation of polyclonal anti-LF antibodies and MoAb AGM 2.29 with molar excess of soluble LF resulted in a marked inhibition of binding to activated neutrophils (Fig. 3). By contrast, no inhibition was found by preincubating polyclonal anti-LF and MoAb AGM 2.29 with molar excess of negative antigen control BSA. These experiments demonstrated that the binding of both polyclonal anti-LF and MoAb AGM 2.29 was specific.

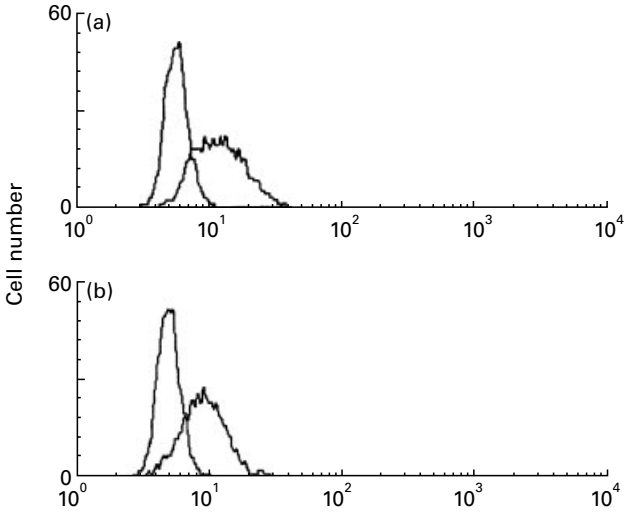

Fig. 1. Priming of freshly drawn blood granulocytes with phorbol myristate acetate (PMA). In each panel, the left histogram represents the mean fluorescence intensity (MFI) obtained with normal mouse immunoglobulin. Increase in MFI was obtained both with polyclonal anti-lactoferrin (LF) antibodies (a) and with MoAb AGM 2.29 (b).

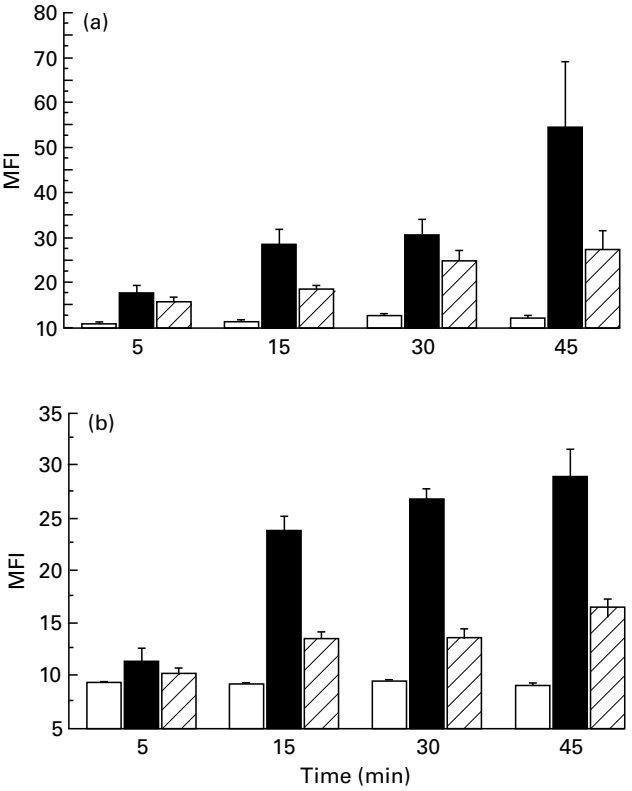

Fig. 2. Lactoferrin (LF) expression on freshly drawn blood granulocytes after 5, 15, 30 and 45 min of priming with phorbol myristate acetate (PMA) (a) and tumour necrosis factor-alpha (TNF-α) (b). □, Mean fluorescence intensity (MFI) obtained with normal mouse immunoglobulin; ■, MFI obtained with polyclonal anti-LF antibodies; ▨, MFI obtained with MoAb AGM 2.29.

Effect of sequential dextran sedimentation and Lymphoprep density gradient centrifugation on LF expression

The isolation of granulocytes by sequential dextran sedimentation and Lymphoprep density gradient centrifugation resulted in the surface expression of LF. In fact, an increase in MFI was demonstrated on neutrophils stained with polyclonal anti-LF and, to a lesser extent, on those stained with AGM 2.29. Table 1 shows the representative results obtained in five healthy donors. No increase in MFI was demonstrated when isolated neutrophils were stained with AGM 10.14. When purified granulocytes were incubated with PMA or TNF-α, irrelevant further expression of LF was observed (data not shown).

Staining of granulocytes with MoAbs following membrane permeabilization

As MoAb AGM 10.14 failed to bind surface LF, membrane permeabilization was performed in order to ascertain the binding of anti-LF MoAbs to cytoplasmic LF. Following membrane

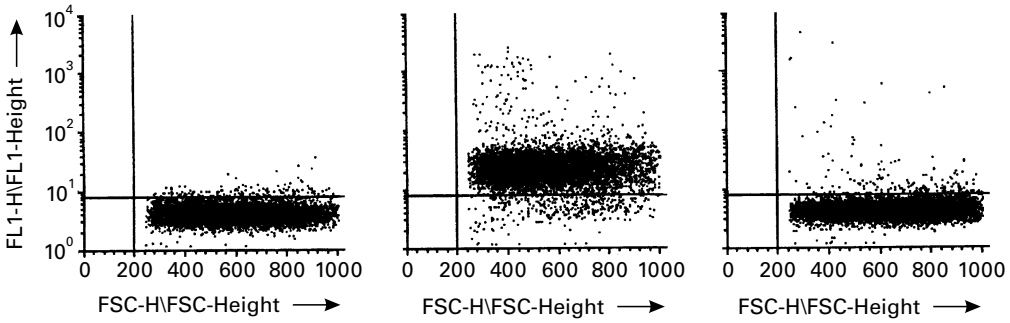

Fig. 3. Inhibition of polyclonal anti-lactoferrin (LF) antibodies binding to granulocytes by soluble LF, evaluated in a dot plot representation (fluorescence (FL1) *versus* forward scatter (FSC)): in the left panel the control obtained with normal mouse immunoglobulin; the middle panel represents the binding of polyclonal anti-LF antibodies to granulocytes, the right panel shows the almost complete inhibition obtained after preincubation of polyclonal anti-LF with molar excess of soluble LF.

permeabilization, a marked increase in MFI was obtained staining granulocytes with both anti-LF MoAbs. This finding demonstrated that AGM 10.14 and AGM 2.29 were able to bind intracytoplasmic LF (Fig. 4).

Indirect immunofluorescence analysis of MoAb reactivity with human neutrophil granulocytes

To analyse the reactivity of the anti-LF MoAbs with the cytoplasmic granules of human neutrophil granulocytes, indirect immunofluorescence (IIF) experiments were performed. As shown in Fig. 5, AGM 2.29, AGM 10.14 and polyclonal antiserum reacted with human neutrophils, the IIF pattern being diffuse granular cytoplasmic (C-ANCA) with central accentuation on formalin–acetone-fixed cells. MoAbs and polyclonal antiserum produced a perinuclear pattern (P-ANCA) on ethanol-fixed PMN.

DISCUSSION

In recent years anti-LF antibodies have been a frequent focus of attention, as they are often found in patients affected by RA [9,10], ulcerative colitis, and primary sclerosing cholangitis [11].

Although evidence for pathogenicity of anti-LF antibodies is much less than for anti-MPO and anti-PR3 autoantibodies, Mulder *et al.* have recently reported that anti-LF ANCA induce the respiratory burst with superoxide production in primed neutrophils as LF becomes accessible at the cell surface after priming of granulocytes with TNF-α [13]. These results were achieved using polyclonal rabbit anti-LF antibodies, or anti-LF antibodies derived from ANCA-positive patients affected by RA or IBD.

We recently produced and characterized two new MoAbs against human LF as a potential tool for evaluating the surface expression of LF on neutrophil granulocytes. These MoAbs recognize spatially distant epitopes of LF and probably bind 'linear' epitopes since they react in immunoblotting with denatured LF after electrophoretic separation, as described elsewhere [14].

The results of this study show that both polyclonal anti-LF antibodies and MoAbs AGM 10.14 and AGM 2.29 failed to stain 'resting' neutrophils, thus indicating that LF is not constitutively expressed on the granulocyte surface. Indeed, to minimize the possibility of neutrophil activation following the experimental procedure, we performed preliminary experiments on whole

Table 1. Isolation of granulocytes of five healthy donors by sequential dextran sedimentation and Lymphoprep density gradient centrifugation

Case	NMS	Anti-LF	AGM 2.29	AGM 10.14
1	5·35	46·86	13·6	5·4
2	3·8	34	10	5
3	6·9	66·8	13	4·4
4	3·5	29·5	16·6	5·2
5	6·5	53·4	18·5	5·9
Mean ± s.d.	5·2 ± 1·5	46·11 ± 15	14·3 ± 3·3	5·1 ± 0·5

An increased mean fluorescence intensity (MFI) was demonstrated on neutrophils stained with polyclonal anti-lactoferrin (LF) and, to a lesser extent, with AGM 2.29. No differences in MFI were found when isolated neutrophils were stained with AGM 10.14 or with normal mouse serum (NMS).

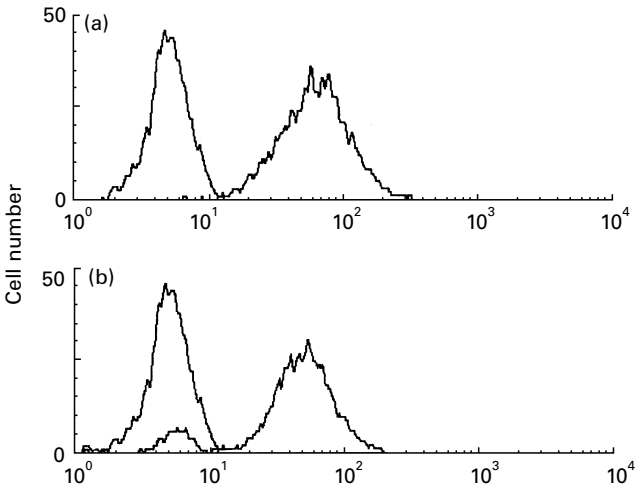

Fig. 4. Staining of granulocytes with AGM 2.29 (a) and AGM 10.14 (b) (right histograms), following membrane permeabilization. In each panel the left histograms represent the mean fluorescence intensity (MFI) obtained with normal mouse immunoglobulin.

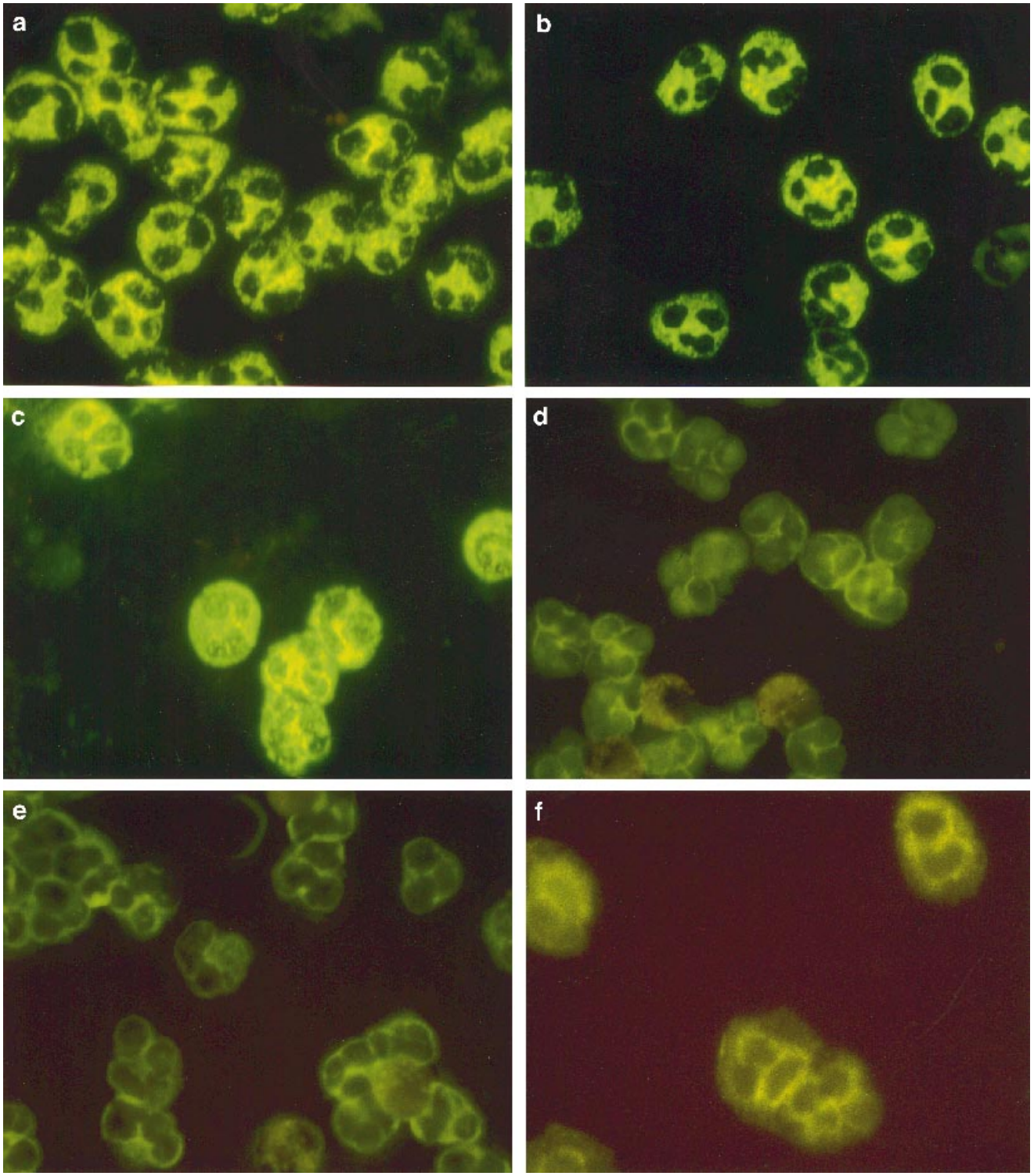

Fig. 5. AGM 2.29 (a), AGM 10.14 (b) and polyclonal antiserum (c) show a diffuse granular cytoplasmic (C-ANCA) with central accentuation on formalin–acetone-fixed cells. AGM 2.29 (d), AGM 10.14 (e) and polyclonal antiserum (f) produced a perinuclear pattern (P-ANCA) on ethanol-fixed PMN.

blood incubated, immediately after venepuncture, with ice-cold buffer with the addition of sodium azide.

Keogan *et al.* have demonstrated an increase in MFI on neutrophils allowed to sediment by gravity (15 min at 37°C) and then stained with $F(ab')_2$ fragments prepared from C-ANCA- and P-ANCA-positive human sera [21]. In this regard, the authors argued that neutrophil activation could be due to the effect of ANCA $F(ab')_2$ preparations used to stain the cells, but they did not formally exclude the possibility that simple gravity sedimentation could be responsible for ANCA antigen expression.

On the other hand, our results are in agreement with those of Falk *et al.* who were unable to detect binding of ANCA immunoglobulins to the surface of unprimed neutrophils by flow cytometry [12]. It should be noted that in this study we demonstrated an increase in MFI on neutrophils stained with polyclonal anti-LF or AGM 2.29 (but not with AGM 10.14), even after isolation of granulocytes by sequential dextran sedimentation and Lymphoprep density gradient centrifugation, without priming with PMA or TNF-α. In addition, the incubation of dextran-isolated granulocytes with the above mentioned activators did not result in a further enhancement of LF expression. These results suggest that when studying the constitutive expression of activation markers, granulocyte isolation by dextran sedimentation and Lymphoprep density gradient centrifugation should be avoided, as it can induce *per se* surface expression of activation molecules. Our findings are in good agreement with previous data reported by other investigators [22–24].

In this study we found that the priming of freshly drawn blood granulocytes with PMA or TNF-α resulted in an increase in MFI, both staining neutrophils with polyclonal anti-LF and, to a lesser extent, with AGM 2.29. This confirms that LF becomes accessible at the cell surface after stimuli and may represent a marker of neutrophil activation. Although we did not use $F(ab')_2$ fragments of anti-LF, the specificity of the binding was demonstrated in experiments in which the preincubation of both polyclonal anti-LF and AGM 2.29 with molar excess of soluble LF resulted in an almost complete inhibition of binding to activated neutrophils.

In all experiments performed in this study, at no time was an increase found in MFI on primed neutrophils stained with MoAb AGM 10.14, which indicates that the LF epitope recognized by this MoAb is not exposed at the cell surface. Given the lack of expression of the epitope recognized by AGM 10.14 in all the experimental conditions we followed, it is probable that LF is always accessible in the same way at the cell surface, i.e. exposing the same epitopes.

On the other hand, the staining of permeabilized granulocytes with AGM 2.29 and AGM 10.14 clearly indicates that both MoAbs react with intracytoplasmic LF. The reactivity with human neutrophils is documented also by IIF: the pattern was diffuse granular cytoplasmic with central accentuation (C-ANCA) on formalin–acetone-fixed cells, and perinuclear (P-ANCA) on ethanol-fixed PMN, thus confirming that ethanol fixation leads to an artefactual redistribution of the antigens recognized by ANCA [10,25].

In conclusion, the results we achieved utilizing anti-LF polyclonal antibodies and MoAb AGM 2.29 demonstrate that in healthy subjects LF is not constitutively expressed on membrane surfaces of circulating neutrophils. LF, however, becomes accessible at cell surface after stimuli, and represents a reliable marker of granulocyte activation. In addition to polyclonal anti-LF preparations, MoAb AGM 2.29 could be utilized as standardized reagent to study the LF expression on neutrophils from patients with different forms of ANCA-positive immunomediated diseases. In this respect, studies of LF expression on granulocytes present at the site of immunoinflammation (e.g. synovial fluid of patients with RA) are in progress in our laboratories. Finally, MoAb AGM 2.29 could represent a useful tool in evaluating the effect(s) and possible pathogenic role of anti-LF antibodies.

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