Immunology 1995 **86** 651–660

Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation

J. JONES & B. P. MORGAN *Department of Medical Biochemistry, University of Wales, College of Medicine, Cardiff, UK*

SUMMARY

Human polymorphonuclear leucocytes (PMN) express proteins that protect them from damage by homologous complement. Protection may be particularly important when these cells migrate to inflammatory sites where complement activation is taking place. Resolution of inflammation involves removal of these PMN. The major mechanism of removal is likely to involve PMN apoptosis followed by recognition and engulfment by macrophages. However, little attention has been paid to the possible relevance of apoptosis to PMN susceptibility to immune effectors. Here we describe a reduction in cell surface expression of two complement regulatory proteins, CD59, an inhibitor of the membrane attack complex and CD55 (decay accelerating factor), an inhibitor of the C3/C5 convertase, on a subpopulation of PMN aged in culture. Loss of these proteins, both attached to the membrane by glycosyl phosphatidylinositol (GPI) anchors, correlated closely with the appearance of apoptotic morphology. We also observed a marked reduction in expression of the GPI-anchored molecule CD16 on apoptotic PMN. Reduced expression of membrane proteins was not confined to those anchored through GPI—several transmembrane molecules including CD11a, CD11b and CD18 were also reduced on apoptotic PMN, whilst others were little changed (CD35, CD46). The precipitous fall in CD16 surface expression on PMN was not specific for apoptosis—*in vitro* incubation of PMN with lipopolysaccharide-inhibited apoptosis but caused a reduction in CD16 expression to 'apoptotic' levels.

INTRODUCTION

Human polymorphonuclear leucocytes (PMN) are the primary effector cells in acute inflammation and have been implicated in the pathogenesis of a large variety of diseases.[1] They are rapidly recruited from the bloodstream in large numbers through the vascular endothelium to the inflamed site.[2] Here they play a vital role in the resolution of inflammation by phagocytosing and destroying micro-organisms and cell debris. However, they can also perpetuate the inflammatory process by releasing a variety of agents with the capacity to cause tissue injury. For efficient resolution of inflammation a fine balance must be struck between the need for clearance of the inflammatory stimulus and the possibility of damage to the host tissues. PMN are inherently short-lived with a half-life of only about 6 hours[3] and the release of enzymes and other components from dying PMN could further exacerbate inflammation. *In vitro* and in several situations *in vivo* PMN have been shown to die by apoptosis, followed *in vivo* by engulfment by tissue macrophages,[4] a process that does not induce release of inflammatory mediators from either cell.[5]

We are particularly interested in the role of complement in activation of PMN and in the initiation and perpetuation of inflammation in the rheumatoid joint. Studies have confirmed that complement activation is ongoing in the joint in rheumatoid arthritis as evidenced by changes in the levels of individual complement components[6,7] and complement activation products[8–10] in rheumatoid synovial fluid. Furthermore, C3 and the membrane attack complex (MAC) have been localized in the rheumatoid synovium.[11,12] Human cells are protected from complement damage by several membrane inhibitors including CD59, CD55 (decay accelerating factor, DAF), CD46 (membrane cofactor protein, MCP) and CD35 (complement receptor 1, CR1) (reviewed in ref. 13) and we have previously shown that CD55 and CD35 are up-regulated on PMN found in synovial fluid from patients with rheumatoid arthritis.[14] However, it is not known whether the expression of the complement regulatory proteins is maintained when PMN undergo apoptosis. Loss of complement regulatory molecules would leave PMN susceptible to lysis in the complement-activating environment of an inflammatory site, causing the release of PMN contents and perpetuation of inflammation. In order to address this possibility we chose to examine the

Received 8 March 1995; revised 19 June 1995; accepted 1 August 1995.

Abbreviations: GPI, glycosyl phosphatidylinositol; PMN, polymorphonuclear leucocyte; MAC, membrane attack complex of complement; PE, phycoerythrin; PI, propidium iodide.

Correspondence: Dr Jane Jones, Department of Medical Biochemistry, 3rd Floor Tenovus Building, Heath Park, Cardiff, CF4 4XX, UK.

expression of complement regulatory molecules and other cell surface markers on PMN aged *in vitro*. We here show that levels of two complement regulatory molecules are markedly diminished on aged PMN and that these changes closely mirror the passage of these cells into apoptosis. Both of these molecules are linked to the membrane by a glycosyl phosphatidylinositol (GPI) anchor and expression of another GPI-anchored molecule, CD16, is also markedly decreased on apoptotic cells suggesting the possibility that GPI-anchored molecules are preferentially lost from cells undergoing apoptosis. However, some trans-membrane proteins are also lost from PMN undergoing apoptosis, indicating that the phenomenon is not unique to GPI-anchored proteins.

MATERIALS AND METHODS

Cell preparation

PMN were isolated from heparinized blood obtained by venepuncture of healthy volunteers as previously described.[15] Briefly, leucocytes were separated from erythrocytes by dextran sedimentation using 0·6% w/v dextran (Fisons, Loughborough, UK). The leucocyte-rich upper layer was then fractionated by layering on HistoPaque (Sigma, Poole, Dorset) followed by centrifugation at 220 *g* for 25 min at room temperature. Residual erythrocytes in the PMN-rich cell pellet were removed by hypotonic lysis. Cell viability was assessed either by exclusion of the nucleic acid strain, propidium iodide (PI) (1 μg/ml) measured on the FACScan (Becton Dickinson, Oxford, UK) or by exclusion of the vital stain trypan blue.

Cell culture

PMN were cultured in RPMI containing 2 mM glutamine, 2 mM sodium pyruvate (Life Technologies, Paisley, UK) and 10% heat-inactivated autologous serum at a density of 1×10^6/ml in 25 cm^3 tissue culture flasks (Nunc, Life Technologies). In some experiments lipopolysaccharide (LPS) from *Escherichia coli* (10 μg/ml) (Sigma L4130) was added to inhibit apoptosis.[16] In one experiment α-1 antitrypsin (1 mg/ml) (Sigma) was added to inhibit the effect of extracellular proteases that may have been released from the PMN. Cells were harvested by gentle agitation and scraping to remove adherent cells. Cell recovery after 20 hr was typically 90%. Cytocentrifuge preparations of cells were stained with Wright's stain and assessed for morphological changes characteristic of apoptosis (nuclear condensation and cell shrinkage) using a × 40 objective.

Antibodies and immunofluorescence staining of cells

CD59 (BRIC 229), CD55 (BRIC 216) and CD58 (BRIC 5) antibodies were obtained from the International Blood Group Reference Laboratory, Elstree, Herts. CD46 (GB24) antibody was a kind gift from Professor John Atkinson, Washington University School of Medicine, St Louis, USA. CD11a, CD11b, CD16, CD18, CD35, CD45, fluorescein isothiocyanate (FITC)-conjugated CD16 antibodies, FITC-conjugated $F(ab)_2$ rabbit antibody to mouse IgG, phycoerythrin (PE)-conjugated $F(ab)_2$ rabbit antibody to mouse IgG were from Dako Ltd., High Wycombe, Bucks. PE-conjugated $F(ab)_2$ goat antibody to rabbit IgG was from Southern Biotechnology Associates, Inc. (Witney, Oxon, UK). TG1 (CD15), an IgM monoclonal antibody specific for cells of the myeloid lineage[17] was a gift from Professor Peter Beverley (Imperial Cancer Research Technology Ltd.).

For single antigen analysis, antibody labelling of intact cells was performed by using indirect immunofluorescence techniques. Briefly, 2×10^5 cells in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0·1% sodium azide (FACS buffer) were incubated with 100 μl of saturating concentrations of primary antibody for 30 min on ice, washed three times in FACS buffer, then incubated with 100 μl of 1/40 rabbit antibody to mouse IgG conjugated to either FITC or PE for 30 min on ice and washed twice. For dual fluorescence analysis cells were incubated first with in-house unconjugated rabbit polyclonal antibody to either CD59 or CD55, then after washing, incubated simultaneously with goat antibody to rabbit immunoglobulins coupled to PE- and FITC-conjugated CD16.

For analysis of surface marker distribution cells were fixed in 1% paraformaldehyde. Cells for sorting were kept on ice.

Flow cytometric analysis and sorting

Flow cytometric analysis was performed on a FACScan (Becton Dickinson). Cells were sorted on a FACS 440 (Becton Dickinson) and collected on ice to minimize further induction or progression of apoptotic changes.

Complement attack assays on PMN

PMN were prepared from the same donor on three consecutive days and cultured as described above. PMN were harvested at 12, 24 and 38 hr. Uncultured PMN were also used (0 hr). PMN (6×10^6) from each harvest were washed and resuspended in RPMI medium (2×10^6/ml) and labelled with calcein acetoxymethyl ester (Calcein AM—10 μg/ml; Cambridge Bioscience, Cambridge) for 30 min at 37°. Calcein AM passively crosses the cell membrane in an electrically neutral form. Inside the cell it is converted by esterases into a polar fluorescent product that is retained by cells with an intact plasma membrane. The calcein-labelled cells were washed and resuspended in 2·4 ml HEPES buffer (120 mM NaCl, 25 mM HEPES, 4·8 mM KCl, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4 \cdot 7H_2O$, 1·3 mM $CaCl_2 \cdot 2H_2O$, pH 7·4). Cells in triplicate (800 μl) were sensitized with 200 μl TG1 antibody for 30 min at 4°. After washing twice and resuspending in HEPES buffer (200 μl) dilutions of normal human serum (NHS) (200 μl) were added as the source of complement. After incubation for 1 hr at 37°, the cells were centrifuged at 800 *g* for 3 min. Aliquots of supernatant (100 μl in duplicate) were taken from each tube and calcein release into the supernatant was measured in a fluorimeter (Wellfluor, Denley Instruments: absorbance 496 nm, emission 517 nm). Measurements were corrected for background calcein release (cells incubated with 1 : 2 serum but no TG1 antibody) and expressed as percentage cell death (100% measured as the release from cells incubated with 0·1% Triton-X-100).

RESULTS

Expression of multiple cell surface markers on PMN change during ageing *in vitro*

PMN were prepared from peripheral blood by standard methods which gave 94–98% purity. All protocols for isolating PMN from peripheral blood are likely to cause some degree of activation.[18–21] In an effort to minimize activation we have compared different protocols. In our hands the method described yields unclumped cells which appear non-activated and retain basal intracellular calcium levels of approximately 100 nM.

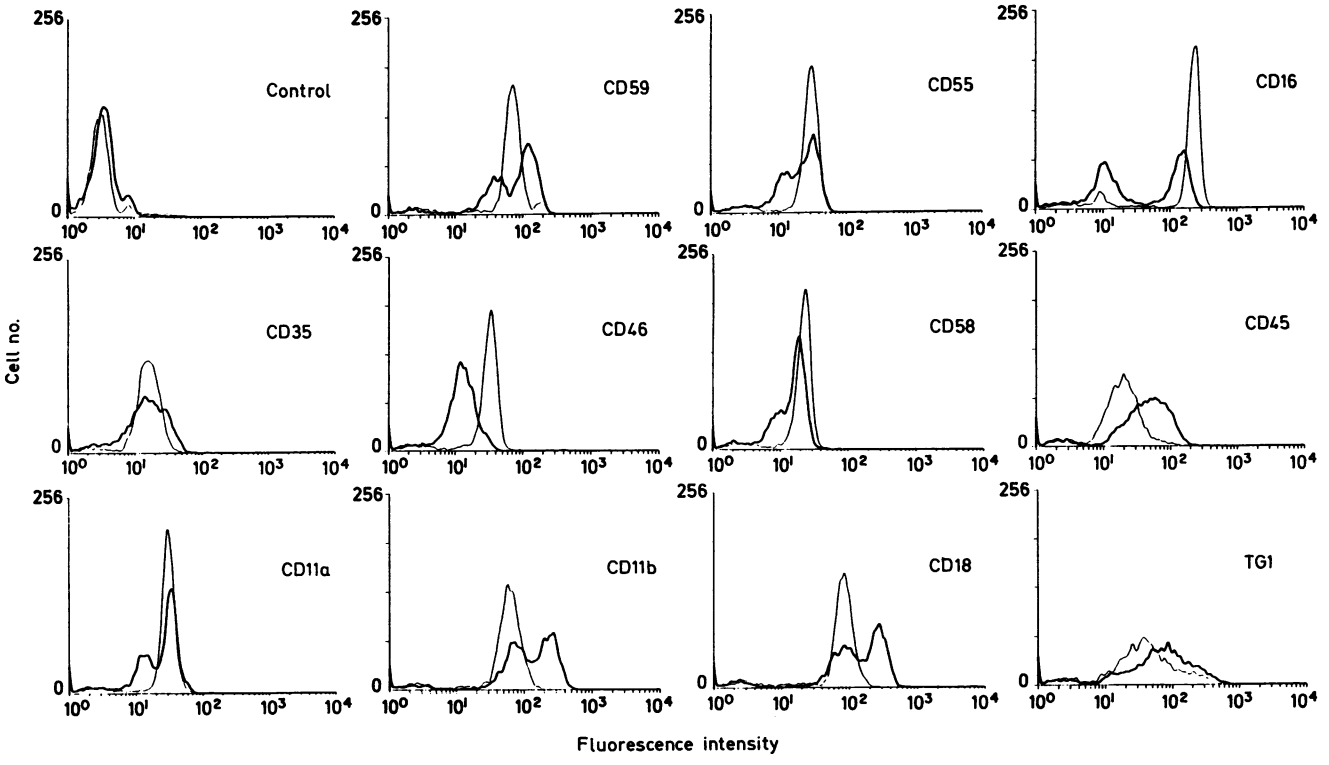

Figure 1. Flow cytometric analysis of fresh and aged PMN populations labelled with antibodies to cell surface antigens. PMN were labelled with a variety of antibodies either immediately after preparation or after 20 hr of culture in RPMI medium containing 10% autologous serum. Single parameter histograms are shown for each cell marker. Faint lines represent the fresh population and bold lines represent the aged PMN. At 20 hr the percentage of apoptotic cells (defined morphologically) was 38%. Fresh and aged PMN were greater than 95% viable as assessed by trypan blue and PI exclusion.

Table 1. Function of antigens investigated in this study, their mode of membrane attachment and their relative expression on bright and dim subpopulations of aged PMN

CD No.	Other name(s)	Function	Anchor	Ratio (bright : dim)
CD59	HRF 20, MIRL Protectin	Inhibits MAC formation	GPI	5 : 1
CD55	Decay-accelerating factor (DAF)	C3 convertase inhibitor	GPI	2 : 1
CD46	Membrane cofactor protein (MCP)	Degradation of C3b and C4b	TM	n/a
CD35	Complement receptor 1 (CR1)	Inhibits classical and alternative path	TM	n/a
CD16	Fcγ RIII	Binds aggregated IgG	GPI	10 : 1
CD11a	LFA-1	Cell/cell and cell/ matrix interaction	TM	2·5 : 1
CD11b	Complement receptor 3 (CR3)	Binds iC3b	TM	3·3 : 1
CD18	Integrin B chain	Common chain for integrin family	TM	3·3 : 1
CD45	—	Tyrosine phosphatase	TM	2·5 : 1
CD58	LFA-3	Binds CD2	GPI	2 : 1
CD15	TG1	Branched pentasaccharide	n/a	n/a

The results are expressed as the ratio of peak channel fluorescence for the bright and dim plopulations for each antigen.

GPI, glycosyl phosphatidylinositol; TM, transmembrane; n/a, not applicable (only one fluorescence peak seen).

Freshly prepared PMN showed homogeneous staining for all surface markers used in this study with only a single peak apparent for each marker (Fig. 1). In contrast, when PMN were cultured for 20 hr at 37° in 5% CO_2, a distinct bimodal distribution of many cell markers was apparent. This bimodal distribution was clearest for CD16 which showed two well-separated fluorescent peaks, while for several markers (CD11a, CD11b, CD18, CD55, CD58 and CD59) two peaks were clearly apparent but not completely separated. For other markers (CD45) there was a shoulder on the left side of the main peak and for some (CD35 and CD46) only one peak was seen (Fig. 1). For most of the markers where two populations were found (CD11a, CD16, CD55), one population of aged cells retained a level of expression similar to that of fresh cells while the second population expressed a reduced level. In contrast, the expression of some markers (CD11b, CD18) was increased on a subpopulation of aged cells compared to fresh cells while there was no change in the second population. CD59 expression was unique in that the level was approximately doubled on one subpopulation and reduced by 50% on the second population of aged cells compared to fresh cells.

The results shown in Fig. 1 are from a single donor but cell surface marker analysis on PMN from three other normal donors all showed similar patterns to those in Fig. 1, though the relative percentage of cells in the bright and dim peaks varied between individuals. Table 1 summarizes the data from these four donors expressed as a ratio of peak fluorescence intensity of bright cells to that of dim cells in the aged population. Such an analysis was only possible when two fluorescence peaks were clearly defined. The largest difference between expression on bright and dim cells was for CD16, with the dim subpopulation expressing only 10% of that expressed on bright cells. CD59 expression on dim cells was only 20% that of bright cells. For CD11a, CD11b, CD18, CD55, CD45 and CD58 the difference between subpopulations was less pronounced with dim cells expressing between 30 and 50% of that on bright cells. For those antigens where only one fluorescent peak was observed, this peak was wider than for fresh cells (CD15, CD35, CD46), suggesting a more heterogeneous expression of these markers on aged cells.

In a preliminary attempt to address the mechanism(s) by which markers are lost from a subpopulation of PMN during ageing the protease inhibitor α-1 antitrypsin was added to PMN for the duration of the overnight culture. This naturally occurring plasma component, which inhibits the action of neutrophil elastase and several other proteases,[22] did not prevent the loss of cell surface markers (results not shown).

The level of expression of CD59 and CD55 correlates with apoptotic morphology

To determine whether there was any correlation between the reduction in expression of the GPI-anchored complement regulatory molecules, CD59 and CD55, and morphology of the cells, PMN populations that had been cultured for 20 hr *in vitro* were labelled with either CD55 or CD59 antibodies followed by PE-conjugated rabbit antibody to mouse immunoglobulin.

Sort gates were set at the top (peak channel) of each fluorescent peak on the FACS440 to sort the brightest from the dimmest cells. When cytocentrifuge preparations were made from the sorted cells and apoptosis estimated by morphology, it was apparent that sorting on the basis of expression of CD59 or CD55 efficiently separated apoptotic from non-apoptotic PMN. In each case the dim cells were greater than 80% apoptotic while the bright cells were greater than 98% non-apoptotic (Table 2; Fig. 2).

Table 2. Apoptosis in PMN sorted on the basis of cell surface antigen expression

Antibody		% Apoptotic Donor 1	% Apoptotic Donor 2
CD59	B	0	0
	D	80	93
CD55	B	1	1
	D	84	97
CD16	B	1	1
	D	91	95
CD11a	B	1	1
	D	89	97
CD11b	B	4	0
	D	60	89
CD18	B	1	4
	D	89	82
CD58	B	11	4
	D	48	62
CD15	B	4	4
	D	69	88

Aged PMN (20 hr in culture) were stained for various cell surface antigens and sorted on a FACS440 on the basis of fluorescence intensity. Cells were >95% viable as assessed by their ability to exclude trypan blue. Where there were two clear peaks, the sort gates were set around the peaks. Where distinct peaks were less clearly defined, the brightest cells were sorted from the dimmest cells as described in the text. Cytocentrifuge preparations were made from the sorted cells, stained with Wright's stain and the percentage of apoptotic cells counted. At least 500 cells were counted per slide. Results are presented for two individuals. B, bright; D, dim.

The level of expression of some other GPI-anchored molecules also correlates with apoptotic morphology

The correlation between apoptotic morphology and expression of two GPI-anchored molecules, CD16 and CD58, not involved in complement regulation was also examined by sorting. For CD16, sort gates were easily defined around the two well-separated fluorescent peaks while for CD58 the sort gates were set at the peak channels of the two closely adjacent peaks. CD16 bright cells were 99% non-apoptotic and CD16 dim cells greater than 90% apoptotic (Fig. 2). CD58 bright cells were also predominantly non-apoptotic (>88%); however, CD58 dim cells were a mixture of non-apoptotic and apoptotic cells (48–62% apoptotic) (Table 2).

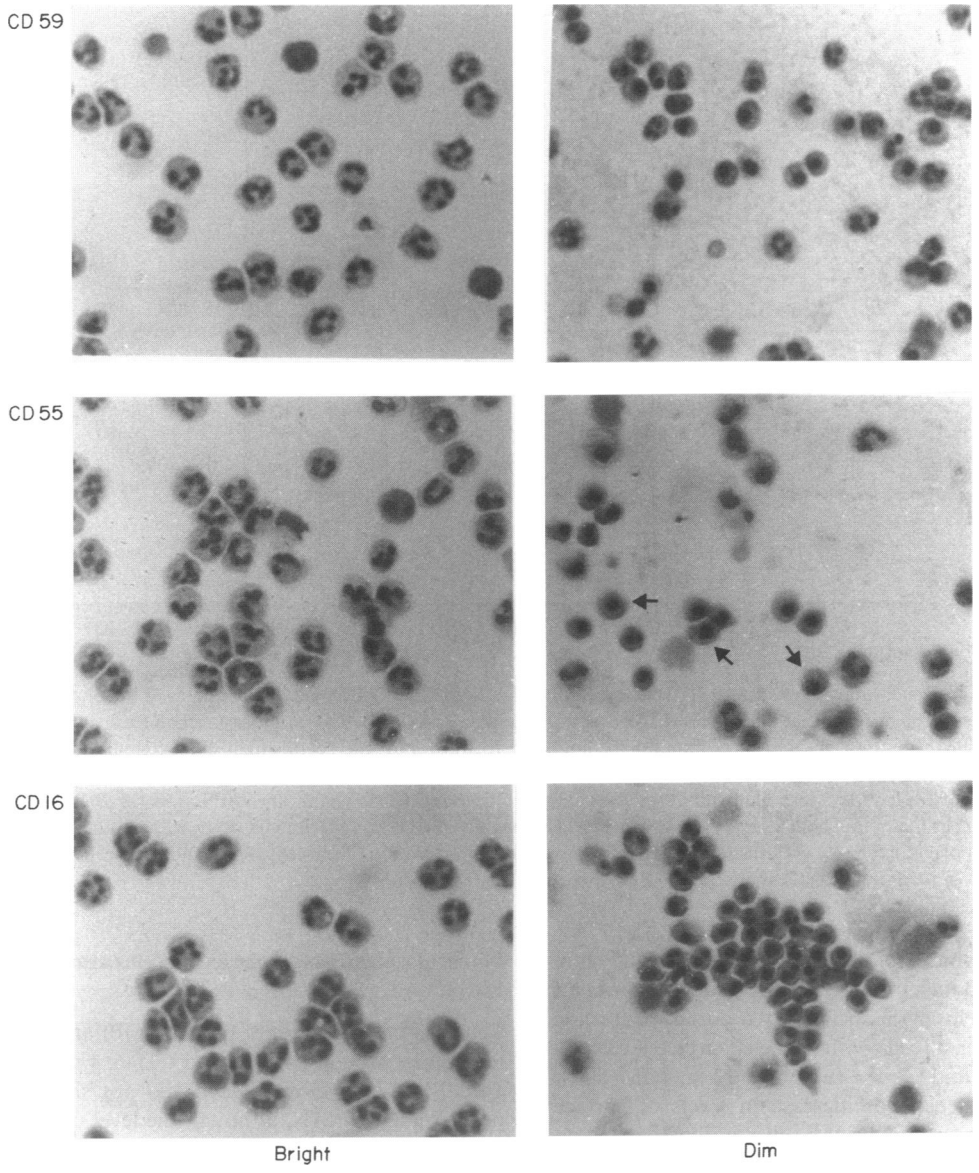

Figure 2. Cytocentrifuge preparations of PMN sorted on the basis of fluorescence intensity for CD59, CD55, or CD16. PMN cultured for 20 hr were labelled for CD59, CD55, or CD16 by using BRIC 229, BRIC 216 or Dako anti-CD16 antibodies respectively, in combination with PE anti-mouse IgG. A FACS440 cell sorter was used to separate those PMN with high levels of expression of these antigens (bright) from those with low levels (dim). Cytocentrifuge preparations of the sorted cells, stained with Wright's stain, are shown. Several apoptotic cells are indicated by arrows. Magnification ×400.

Loss of CD16 occurs in parallel with loss of CD59 and CD55

The above data, by demonstrating that apoptotic cells are dim and non-apoptotic cells bright for each of the markers CD16, CD55 and CD59, indicate that loss of these three markers occurs together during passage into apoptosis. In order to confirm this, aged PMN were double-stained for CD16 and either CD59 or CD55 and the cells analysed on the FACScan. The forward and side light scatter characteristics of fresh PMN and aged PMN (20 hr in culture) were different and the cells included in the fluorescence analysis are highlighted (Fig. 3, a and d). Aged PMN that were bright for CD16 were also bright for CD59 (R2 Fig. 3e) and CD55 (R2 Fig. 3f), while cells that expressed lower levels of CD16 also expressed lower levels of CD59 (R3 Fig. 3e) and CD55 (R3 Fig. 3f). Fresh PMN were uniformly bright for all three markers (Fig. 3b and 3c). The small percentage (< 5%) of fresh cells that expressed reduced levels of CD16, CD59 and CD55 were shown by sorting to be contaminating eosinophils (data not shown).

Temporal changes in CD59, CD55 and CD16

To investigate the temporal relationship between changes in cell surface expression of CD59, CD55 and CD16 and the appearance of morphological features of apoptosis, time course experiments were undertaken. The results from PMN cultured for 0–29 hr are shown in Table 3 and the data from

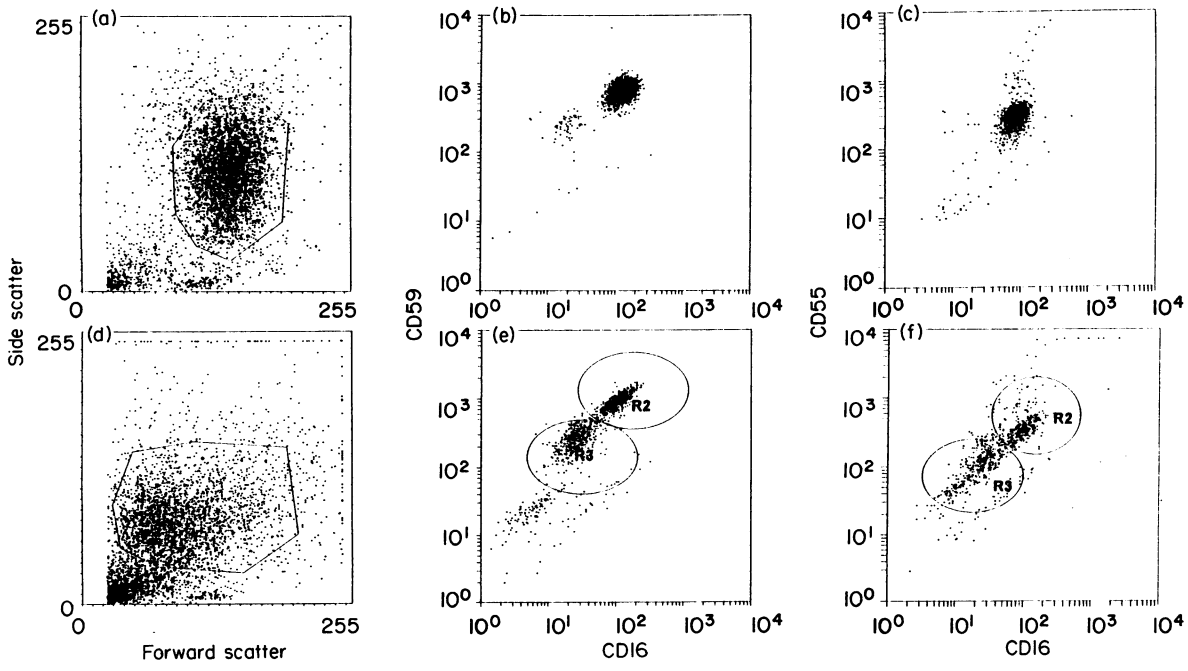

Figure 3. Dual fluorescence analysis of surface CD16 expression and either CD59 or CD55 on fresh and aged PMN. Coexpression of the GPI-linked complement regulatory molecules CD59 and CD55 with GPI-linked CD16 was measured on fresh and aged (cultured for 20 hr at 37°) PMN by dual fluorescence immunolabelling. The forward and side scatter light characteristics of the fresh and cultured cells included in the analysis are shown in (a) and (d) respectively by the enclosed region. The dual fluorescence dot displays show that >95% of fresh PMN were uniformly bright for CD16, CD59 and CD55 (b, c). On PMN aged for 20 hr in culture, the subpopulation of cells that retain high levels of CD16 also retain high levels of CD59 (e, R2) and CD55 (f, R2) while a loss in CD59 and CD55 expression correlates closely with the loss of CD16 on a second subpopulation of the cells (R3, e and f respectively). The results are representative of three individual experiments.

three of these time points are displayed graphically in Figure 4. The time course data strongly suggest that the onset of PMN apoptosis is closely associated temporally with the increase in the number of cells with reduced surface levels (% dim) of CD59, CD55 and CD16. Table 3 and Fig. 4 also show that there is a time-dependent increase in levels of surface CD59 on the non-apoptotic bright cells, a time-dependent decrease in CD16 on these same cells, and no change in CD55.

Levels of some transmembrane proteins also change on apoptotic PMN

The level of expression of two non-GPI anchored complement regulatory proteins, CD35 and CD46, was also measured on aged PMN. CD35 and CD46 did not show two fluorescent peaks on aged PMN, although the level of CD46 was slightly decreased on aged cells compared to fresh cells. However, for other transmembrane molecules, CD11a, CD11b and CD18 and, with some donors, the myeloid-specific marker CD15, a

Table 3. Temporal changes in CD59, CD55 and CD16

	CD59		CD55		CD16		
Time (hr)	% Dim	Bright peak	% Dim	Bright peak	% Dim	Bright peak	Morphological Apoptosis (%)
0	12	111	0	22	15	255	5
2	10	115	1	25	13	246	3
5	8	138	5	29	12	237	10
9	9	149	4	26	6	213	7
23	25	172	19	25	28	184	19
29	36	172	28	25	40	124	37

PMN aged in culture for 0–29 hr were stained for CD59, CD55 and CD16 surface expression at various times and analysed by flow cytometry. As two fluorescence peaks were seen, the results are expressed as the percentage of cells in the lower fluorescence peak (% dim) and as the peak channel number of the brighter peak (bright peak). Morphological apoptosis (%) was measured by counting at least 500 cells on a cytocentrifuge slide preparation.

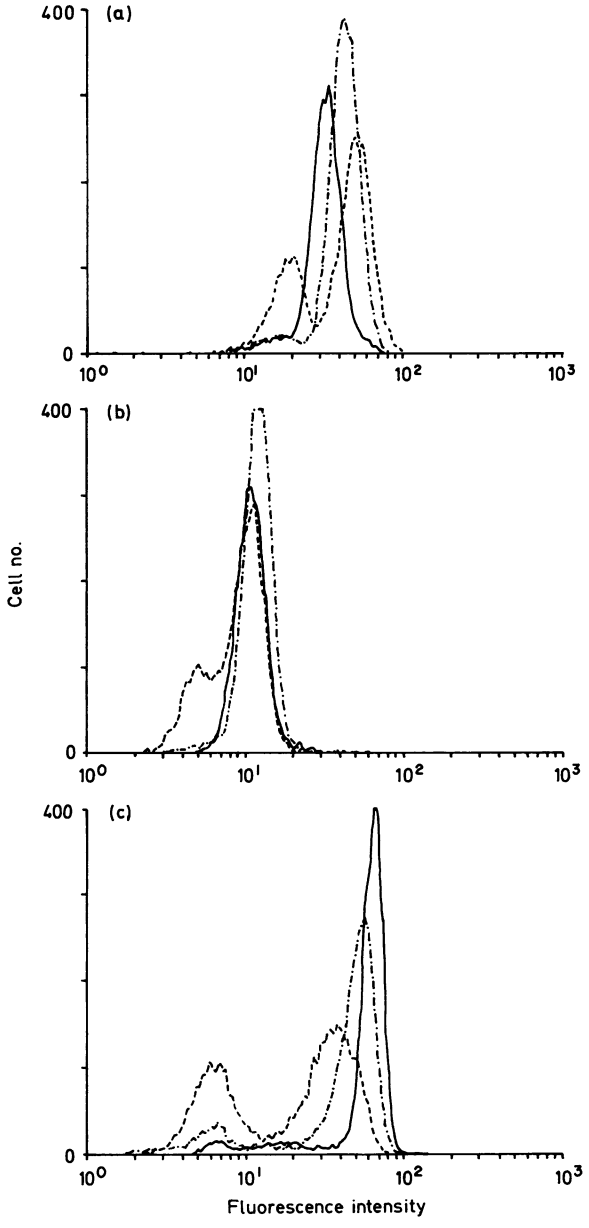

Figure 4. Time course of changes in expression of CD59, CD55 and CD16 during *in vitro* culture of PMN. Freshly isolated PMN (——) or neutrophils that had been cultured for 9 (– · – · –) or 29 (– – –) hr were examined for surface expression of (a) CD59, (b) CD55 and (c) CD16. Expression was determined by indirect immunofluorescence and flow cytometry.

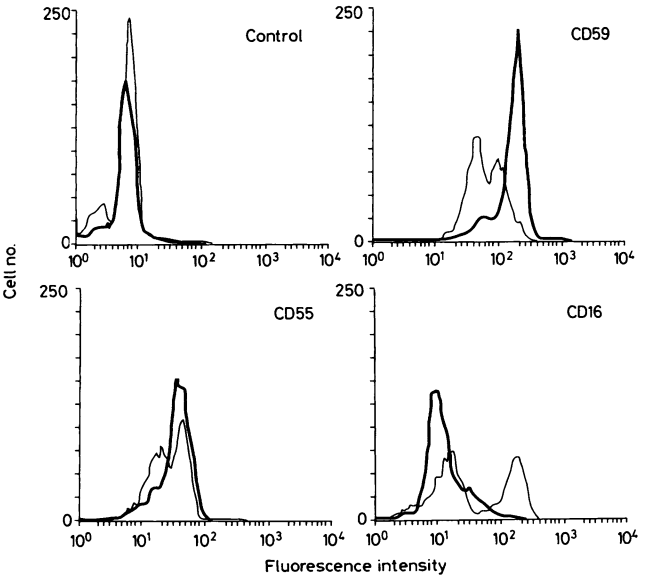

Figure 5. Effect of LPS addition on PMN expression of CD59, CD55 and CD16. PMN were cultured for 20 hr with or without LPS (10 μg/ml) in RPMI containing 10% autologous serum. The cultured cells were labelled with antibodies to either CD59, CD55, or CD16, in combination with FITC anti-mouse IgG. The histograms shown were obtained on the FACScan. Faint lines and bold lines represent cells from the same donor cultured for 20 hr in the absence or presence of LPS respectively. The percentage of apoptotic cells was determined by morphological changes and was 44% in the absence of LPS and 14% in the presence of LPS.

bimodal distribution was apparent. Therefore aged (20 hr) PMN populations were labelled for either CD11a, CD11b, CD18, or CD15 and sorted as above on the basis of fluorescence. Table 2 shows that over 95% of CD11a, CD11b, CD18 and CD15 bright cells were non-apoptotic PMN, whilst the majority of dim cells were apoptotic.

Loss of CD16 expression on PMN is induced by the apoptosis inhibitor LPS

Lipopolysaccharide was added to overnight cultures of PMN to inhibit apoptosis.[16] The morphology and phenotype of aged LPS-treated and untreated PMN was compared. Examination of cytocentrifuge preparations of cells from these cultures showed that LPS at 10 μg/ml reduced apoptosis from 44% to 14% and 46% to 28% after 20 hr in culture on cells from two donors. Fluorescence profiles of cells labelled with three different GPI-anchored molecules (CD16, CD55 and CD59) showed that CD59 expression increased on non-apoptotic cells during ageing in the presence of LPS, CD55 was little changed, yet CD16 on the majority of cells was precipitously decreased to levels similar to those found on apoptotic PMN (Fig. 5). When PMN aged in the presence of LPS were sorted on the basis of CD16 expression the CD16 bright population contained only non-apoptotic cells whereas the CD16 dim population contained a mixture, the majority of which were non-apoptotic (results not shown).

The susceptibility of PMN to complement increases with time in culture

To examine the significance of the observed alterations in expression of the complement inhibitory molecules CD55 and CD59 on apoptotic PMN, cells taken from culture at various times after seeding were labelled with the viability indicator dye calcein-AM, sensitized with antibody and attacked with complement. The loss of viability of the cells was determined by their release of calcein.

The results show (Fig. 6) that as the cells aged in culture and a greater proportion became apoptotic, their susceptibility to

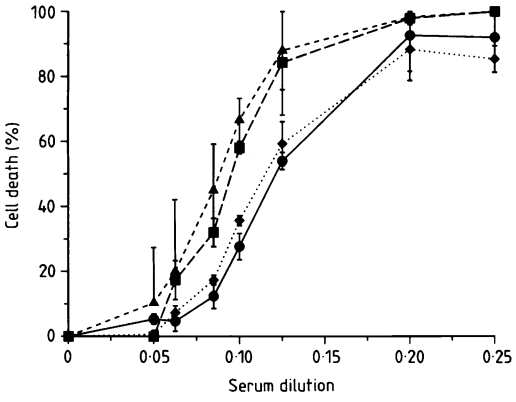

Figure 6. Susceptibility of PMN to complement attack: the effect of *in vitro* cell ageing. PMN cultured for varying times (0–38 hr) were labelled with calcein, sensitized with TG1 antibody and incubated with varying dilutions of NHS. Loss in viability was assessed by calcein release. 0 hr (solid circles); 15 hr (solid diamonds); 24 hr (solid squares); 38 hr (solid triangles) in culture. The results shown are the means (± SD) of triplicate tubes and are corrected for background cell death.

complement attack increased. A serum dilution of 1 : 8 caused 50% calcein release in fresh PMN (0 hr, < 5% apoptotic) while progressively less serum was required to mediate the same release from cells aged for 15, 24 and 38 hr in culture (1 : 12 dilution for 38 hr culture containing 49% apoptotic PMN). The use of the calcein-release assay overcomes the variable viability of the cells at the time of labelling as only live cells take up and convert calcein-AM to its fluorescent analogue. Results were corrected for background lysis (lysis in the presence of serum but no TG1 antibody) which was greater for aged than fresh cells.

The calcein-release assay for measurement of PMN viability was preferred to flow cytometric assays based on propidium iodide uptake or calcein retention as PMN lysis in the presence of high serum doses made flow cytometric 'gating' of cells from debris difficult.

DISCUSSION

PMN aged *in vitro* undergo a series of well-characterized morphological changes tyical of apoptosis (nuclear condensation, reduction in cell size). However, the phenotype of apoptotic PMN has not been well characterized. Given the importance of membrane receptors and regulators in the activation and survival of PMN, changes in expression of these molecules during apoptosis might be of considerable relevance to cell survival in the tissues. We are interested in the role of complement in the perpetuation of inflammation in the rheumatoid joint and have suggested that complement deposition on infiltrating PMN might be an important cause of PMN activation and destruction.[23] Altered expression of complement regulators on PMN undergoing apoptosis might influence their susceptibility to complement damage. Here we demonstrate that expression of the GPI-anchored complement regulators CD59 and CD55 was reduced on a subpopulation of PMN aged *in vitro* (Fig. 1). Cell sorting and time course studies revealed that the reduction in expression of each of these markers correlated precisely with apoptosis (Tables 2 and 3, Fig. 4). Expression of another GPI-anchored molecule, CD16, was also much reduced on apoptotic cells but expression of GPI-anchored CD58 corrrelated less strongly with apoptosis. Expression of several transmembrane molecules was also examined on aged PMN. For some (CD11a, CD11b, CD18) two populations of cells were present and the dim cells were predominantly apoptotic, whereas for others (CD35, CD46), no separation into bright and dim populations was observed. However, the best markers for apoptosis were the GPI-anchored molecules CD16 and CD59.

Phenotypic changes on PMN aged in culture have been previously described for CD16 and an unidentified antigen termed Y151[24,25] and while this manuscript was in preparation two groups reported the close association of reduced CD16 expression with apoptosis in PMN.[26,27] These authors found no reduction in expression of three other GPI-anchored molecules, CD55, CD66b and CD58, and were unable to dissociate decreased expression of CD16 from apoptosis, suggesting that loss of CD16 might be a unique and specific marker for PMN apoptosis. We here confirm the loss of CD16 from apoptotic PMN (to less than 10% of the level on non-apoptotic cells). However, we found that expression of two other GPI-anchored molecules, CD55 (in contrast to previous reports) and CD59 (the latter not previously tested) was also markedly reduced on aged cells and correlated precisely with apoptosis.

Examination of susceptibility to complement lysis demonstrated that aged PMN were much more sensitive than were fresh cells (Fig. 6). These findings may suggest that the observed changes in CD59 and/or CD55 render the cells more susceptible to complement. However, there are alternative interpretations of these data. Aged cells are inherently more 'fragile' than fresh PMN, as indicated by the increased background death with increased time in culture, and despite our endeavours to remove this variable by subtraction of appropriate background lysis, increased fragility may still contribute to the observed differences. Further, the sensitizing antibody, TG1 (anti-CD15), binds less well to apoptotic PMN (Table 2). This would have the effect of reducing complement activation on apoptotic cells with a concomitant underestimation of any increased sensitivity to complement.

The mechanism by which CD16, CD55 and CD59 are lost from PMN undergoing apoptosis is uncertain. A characteristic of apoptotic cells is a reduction in size[28] which might account for small changes in cell surface marker expression by flow cytometry but cannot explain the large losses seen for several proteins or the selectivity of these losses. The inclusion of the protease inhibitor α-1 antitrypsin did not inhibit marker loss, suggesting that proteolytic enzymes released from PMN are not responsible, although other protease inhibitors remain to be tested. Other possible routes of loss include shedding, endocytosis, or decreased biosynthetic capacity. We are currently exploring these different possibilities and in particular, whether soluble forms of these molecules are released during apoptosis. Soluble forms of CD35 (sCR1), CD55 and CD59 are found in plasma[29,30] and PMN are the major source of plasma CD35. CD59 and CD55 shed by PMN undergoing apoptosis at inflammatory sites might accumulate locally and suppress further complement activation.

We also show that reduced expression of CD16 is not specific to apoptosis—a precipitous reduction is induced by incubation with LPS, a stimulus which inhibits PMN apoptosis[16] (Fig. 5). LPS prolongs PMN lifespan by delaying

apoptosis and clearance[16,31] and activates enzyme secretion,[32] adherence[33,34] and release of oxygen metabolites.[35,36] LPS mediates many of its effects after binding to LPS-binding protein in fluids and subsequently to the LPS receptor, CD14, which is a GPI-anchored molecule expressed abundantly on monocytes and in lower amounts on PMN.[34,37] We found that addition of LPS to PMN *in vitro* caused a reduction in apoptosis which was associated with a fall in CD16 expression on the majority of cells to levels equivalent to those found on apoptotic cells. Sorting confirmed that the majority of these CD16 dim cells were non-apoptotic. The effects of LPS were specific to CD16 in that it caused a small increase in expression of CD59 and had little effect on the expression of CD55.

In rheumatoid arthritis leucocytes, predominantly PMN, accumulate in synovial fluid. The route of clearance remains uncertain but is likely to be via apoptosis and phagocytosis by synovial tissue and fluid macrophages.[4,33,38] There is abundant evidence for complement activation in the rheumatoid joint[6,8–12] and the marked reduction in expression of two complement regulators on apoptotic PMN (CD59 only 20% and CD55 50% as compared to non-apoptotic aged cells) may render these cells more susceptible to bystander complement damage and lysis. This may be important if complement damage occurs before phagocytosis ensues or if phagocytosis is inefficient. There is evidence that neutrophil activation occurs in synovial fluid in rheumatoid arthritis with release of neutrophil granule constituents.[39] The release of potent oxidative and enzymatic agents by necrosis would contribute to inflammation *in vivo*.

The loss of other surface receptors may also be of functional relevance. CD16 is an important receptor for immune complexes on PMN and a reduction in its expression during apoptosis would reduce immune complex binding capacity. Changes in expression and properties of the integrins is important for function[40] and the changes observed here may render the PMN less responsive to stimuli. The end result of receptor loss might be the adoption by apoptotic cells of an 'anergic' state which would help prevent the release of inflammatory mediators from the neutrophil and the engulfing macrophage.

ACKNOWLEDGMENTS

We thank the Arthritis and Rheumatism Council for financial support through the award of a programme grant (C0091). We also thank J. Fisher and P. Perera for technical assistance with the cell sorting and Dr Graciela Sala-Newby for helpful discussion. BPM is a Wellcome Senior Clinical Research Fellow.

REFERENCES

1. Malech H.D. & Gallin J.I. (1988) Neutrophils in human diseases. *N Engl J Med* **37,** 687.
2. Roitt I., Brostoff J. & Male D. (1985) Adaptive and innate immunity. In: *Immunology* (eds I. Roitt, J. Brostoff & D. Male) p. 1.3. Churchill Livingstone, Edinburgh.
3. Male D., Champion B. & Cooke A. (1987) Inflammation. In: *Advanced Immunology* (eds D. Male, B. Champion & A. Cooke) p.15.7. Gower Medical Publishing, London.
4. Savill J.S., Wyllie A.H., Henson J.E., Walport M.J., Henson P.M. & Haslett C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* **83,** 865.
5. Meagher L.C., Savill J.S., Baker A., Fuller R.W. & Haslett C. (1992) Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B_2. *J Leukocyte Biol* **52,** 269.
6. Ruddy S. & Austen K.F. (1973) Activation of the complement system in rheumatoid synovitis. *Fed Proc* **32,** 134.
7. Rumfeld W.R., Morgan B.P. & Campbell A.K. (1986) The ninth complement component in rheumatoid arthritis, Behcet's disease and other rheumatic diseases. *Br J Rheumatol* **25,** 266.
8. Mollnes T.E. (1985) Quantification of the C3d split products of human complement by a sensitive enzyme-linked assay. *Scand J Immunol* **21,** 607.
9. Morgan B.P., Daniels R.H. & Williams B.D. (1988) Measurement of terminal complement complexes in rheumatoid arthritis. *Clin Exp Immunol* **73,** 473.
10. Peakman M., Lobo-Yeo A., Senaldi G., Nilsson M., Tee D.E.H. & Vergani D. (1987) Quantification of C3d in biological fluids by an enzyme-linked immunosorbent assay. *J Immunol Meth* **104,** 51.
11. Kemp P.A., Spragg J.H., Brown J.C., Morgan B.P., Gunn C.A. & Taylor P.W. (1992) Immunohistochemical determination of complement activation in joint tissues of patients with rheumatoid arthritis and osteoarthritis using neo-antigen-specific monoclonal antibodies. *J Clin Lab Immunol* **37,** 147.
12. Sanders M.E., Kopicky J.A., Wigley F.M., Shin M.L., Frank M.M. & Joiner K.A. (1986) Membrane attack complex of complement in rheumatoid synovial tissue demonstrated by immunofluorescent microscopy. *J Rheumatol* **13,** 1028.
13. Morgan B.P. & Meri S. (1994) Membrane proteins that protect against complement lysis. *Springer Semin Immunopathol* **15,** 369.
14. Jones J., Laffafian I., Cooper A.M., Williams B.D. & Morgan B.P. (1994) Expression of complement regulatory molecules and other cell surface markers on neutrophils from synovial fluid and blood of patients with rheumatoid arthritis. *Br J Rheumatol* **33,** 707.
15. Morgan B.P. & Campbell A.K. (1985) The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium. *Biochem J* **231,** 205.
16. Yamamaoyo C., Yoshida S.-I., Taniguchi H., Qin M.H., Miyamoto H. & Mizuguchi Y. (1993) Lipopolysaccharide and granulocyte colony-stimulating factor delay neutrophil apoptosis and ingestion by guinea pig macrophages. *Infect Immun* **61,** 1972.
17. Beverley P.C.L., Linch D. & Delia D. (1980) Isolation of human haematopoietic progenitor cells using monoclonal antibodies. *Nature* **287,** 332.
18. Fearon D. & Collins L. (1983) Increased expression of C3b receptors on polymorphonuclear leucocytes induced by chemotactic factors and purification procedures. *J Immunol* **130,** 370.
19. Forsyth K. & Levinsky R. (1990) Preparative procedures of cooling and rewarming increase leucocyte integrin expression and function on neutrophils. *J Immunol* **128,** 159.
20. Hamblin A., Taylor M., Bernhagen J. *et al.* (1992) A method of preparing blood leucocytes for flow cytometry which prevents upregulation of leucocyte integrins. *J Immunol Meth* **146,** 219.
21. Haslett C., Guthrie L.A., Kopaniak M.M., Johnston R.B. & Henson P.M. (1985) Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* **119,** 101.
22. Laurell C.B. & Jeppsson J.O. (1975) Protease inhibitors in plasma. In: *The Plasma Proteins* (ed. F. Putnam) Vol. 1, 2nd edn, p. 229. Academic Press, New York.
23. Morgan B.P. (1990) Complement and rheumatic disease. In: *Complement: Clinical Aspects and Relevance to Disease*, (ed. Morgan, B.P.) pp. 130–40. Academic Press, London.
24. Buckle A.M., Jayaram Y. & Hogg N. (1990). Colony-stimulating factors and interferon-gamma differentially affect cell surface

molecules shared by monocytes and neutrophils. *Clin exp Immunol* **81,** 339.

25. Ichinose Y., Hara N., Ohta M. *et al.* (1990) Recombinant granulocyte colony-stimulating factor and lipopolysaccharide maintain the phenotype of and superoxide anion generation by neutrophils. *Infect Immun* **58,** 1647.
26. Dransfield I., Buckle A-M., Savill J.S., McDowall A., Haslett C. & Hogg N. (1994) Neutrophil apoptosis is associated with a reduction in CD16 (FcγRIII) expression. *J Immunol* **153,** 1254.
27. Homburg H.E., de Haas M., von dem Borne A.E., Verhoeven A.J., Reutelingsperger C.P.M. & Roos D. (1995) Human neutrophils lose their surface FcγRIII and acquire annexin V binding sites during apoptosis *in vitro*. *Blood* **85,** 532.
28. Allen P.D., Bustin S.A. & Newland A.C. (1993) The role of apoptosis (programmed cell death) in haemopoiesis and the immune system. *Blood Rev* **7,** 63.
29. Danielsson C., Pascual M., French L., Steiger G. & Schifferli J.A. (1994) Soluble CR1 is released from leucocytes by surface cleavage. *Clin Exp Immunol* **97,** (Abstract 24) 20.
30. Medof M.E., Walter E.I., Rutgers J.L., Knowles D.M. & Nussenzweig V. (1987) Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J Exp Med* **165,** 848.
31. Lee A., Whyte M.K.B. & Haslett C. (1993) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukocyte Biol* **54,** 283.
32. Fittschen C., Sandhaus R.A., Worthen G.C. & Henson P.M. (1988) Bacterial lipopolysaccharide enhances chemoattractant-induced elastase secretion by human neutrophils. *J Leukocyte Biol* **43,** 547.
33. Dahinden C. & Fehr J. (1983) Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. *J Immunol* **130,** 863.
34. Wright S.D., Ramos R.A., Hermanowski Vosatka A., Rockwell P. & Detmers P.A. (1991) Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. *J Exp Med* **173,** 1281.
35. Aida Y. & Pabst M.J. (1991) Neutrophil responses to lipopolysaccharide: effect of adherence on triggering and priming of the respiratory burst. *J Immunol* **146,** 1271.
36. Guthrie L.A., McPhail L.C., Henson P.M. & Johnston Jr. R.B. (1984) Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide: evidence for increased activity of the superoxide-producing enzyme. *J Exp Med* **160,**1656.
37. Wright S.D., Ramos R.A., Tobias P.S., Ulevitch R.J. & Mathison J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249,** 1431.
38. Savill J. (1992) Macrophage recognition of senescent neutrophils. *Clin Sci* **83,** 649.
39. Nurcombe H.L., Bucknall R.C. & Edwards S.W. (1991) Activation of the neutrophil myeloperoxidase-H_2O_2 system by synovial fluid isolated from patients with rheumatoid arthritis. *Ann Rheum Dis* **50,** 237.
40. Zimmerman G.A., Prescott S.M. & McIntyre M.T. (1992) Endothelial cell interactions with granulocytes: tethering and signalling molecules. *Immunol Today* **13,** 93.