Effect of aging on CD11b and CD69 surface expression by vesicular insertion in human polymorphonuclear leucocytes

J. M. NOBLE*, G. A. FORD† and T. H. THOMAS*

*Department of Medicine, University of Newcastle upon Tyne, Claremont Place, Newcastle Upon Tyne NE2 4HH, U.K., and †Department of Clinical Pharmacology, University of Newcastle upon Tyne, Claremont Place, Newcastle Upon Tyne NE2 4HH, U.K.

ABSTRACT

The exocytosis of intracellular vesicles is an important function of the plasma membrane, which is responsible for hormone secretion, cell surface expression of antigens, ion transporters and receptors, and intracellular and intercellular signalling. Human aging is associated with many physiological and cellular changes, many of which are due to alterations in plasma membrane functioning. Alterations in vesicle externalization with age could account for many of these changes. We investigated whether alterations in vesicle exocytosis occur with increasing age by flow-cytometric determination of CD11b and CD69 expression on the surface of human polymorphonuclear leucocytes (PMN) stimulated with phorbol myristate acetate (PMA), a tumour promoter which binds to and activates protein kinase C (PKC) directly, or with formyl-Met-Leu-Phe (fMLP), which activates PKC indirectly via interactions with a cell surface receptor and G-protein, and subsequent inositol phosphate hydrolysis. Following stimulation with PMA, a decrease in the proportion of PMN expressing CD69 at high levels was observed in elderly compared with young subjects (young, 55.3%; elderly, 43.9%; P < 0.01). No aging-related differences in the proportion of PMN expressing CD11b (young, 73.7%; elderly, 68.4%; P > 0.15), or in the number of molecules of CD69 or CD11b expressed per cell, were observed. Stimulation with fMLP or low PMA concentrations resulted in full CD11b expression but minimal CD69 expression in both young and elderly subjects. Cells which expressed CD69 had no CD11b expression, while those cells expressing CD11b had minimal CD69 expression. Thus the PMA-induced expression of CD11b and CD69 in human PMN represents two separate processes, only one of which is affected in aging. CD11b expression appears to require a lesser degree of PKC stimulation compared with that required for CD69 expression. The age-associated reduction in PMA-stimulated CD69 expression may occur either at or distal to PKC activation. Such a decrease may contribute to the age-associated impairments in PMN function that contribute, in turn, to immunosenescence.

INTRODUCTION

Fusion of intracellular vesicles with the plasma membrane is a fundamental process in cells, which leads both to the expression of cell surface receptors, transporters and signalling proteins, and to the secretion of hormones and neurotransmitters. This process depends on an intact and functioning plasma membrane and a range of membrane-associated proteins [1,2]. Several lines of evidence suggest that plasma membrane function is altered as people age. These include changes in transport proteins, hormone–receptor responses [3], immune system signalling [4] and

Key words: aging, antigen expression, CD11b, CD69, polymorphonuclear leucocytes.
Abbreviations: fMLP, formyl-Met-Leu-Phe; PE, phycoerythrin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leucocytes.
Correspondence: Dr T. H. Thomas.
membrane lipid properties [5,6]. Such alterations may affect vesicle fusion with the plasma membrane. We hypothesized that aging is associated with changes in vesicle insertion into the membrane that may compromise cell function.

Fusion of intracellular vesicles is particularly important for polymorphonuclear leucocyte (PMN) adhesion, migration and release of reactive oxygen species. These processes are reduced in PMN from elderly subjects [7]. In order to determine whether aging is associated with alterations in vesicle fusion, we examined the effects of aging on the process of insertion of two antigens into the plasma membrane of human PMN following cell activation in vitro: CD11b, a/b2 integrin essential for PMN adhesion, chemotaxis and phagocytosis [8]; and CD69, an early activation marker whose role in PMN and T-cell activation has yet to be fully elucidated [9]. In quiescent PMN, CD11b and CD69 are stored in intracellular vesicles. Following cell activation, vesicles move to fuse with the cell membrane within min to expose the antigens on the cell surface, a process which in PMN is independent of new protein synthesis. In order to elucidate some of the mechanisms underlying antigen expression in human PMN, PMN were activated with phorbol 12-myristate 13-acetate (PMA), a phorbol ester which binds directly to and activates intracellular protein kinase C (PKC), and with formyl-Met-Leu-Phe (fMLP), a chemotactic peptide which activates PKC indirectly via a cell surface receptor and inositol phosphate hydrolysis.

**METHODS**

**Subjects**

Venous blood was obtained from 21 healthy young subjects (mean age 30 years; range 25–35 years; 11 male) and 17 healthy elderly subjects (mean age 68 years; range 63–77 years; 10 male). Young subjects were recruited from clinical and laboratory staff, and elderly subjects were independent community dwellers. All subjects fulfilled the criteria of the Seneuir protocol [10] for immunogerontological studies. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and was approved by the Newcastle Health Authority/Newcastle University Joint Ethical Committee. Subjects gave written informed consent prior to the study.

Venous blood was collected into heparinized glass tubes and kept on ice prior to use in experiments. The time from venesection to the start of incubations did not exceed 20 min.

**Experimental protocol**

Samples of 200 µl of heparinized whole blood were incubated at 37 °C with 200 µl of Dulbecco’s PBS (pH 7.40), or PBS containing fMLP (from a stock solution in PBS) or PMA (from a stock solution in ethanol; the final ethanol concentration in incubations was 1%, v/v), then cooled on ice. For experiments comparing PMN from young and elderly groups, incubation times of 15 min for fMLP and 30 min for PMA, and concentrations of 0.1 µM fMLP and 100 ng/ml PMA, were chosen to ensure that maximal stimulation of PMN had occurred. Light microscopy of samples following PMA stimulation revealed no evidence of PMN aggregation (results not shown). Following cooling, 100 µl aliquots of blood were stained with 10 µl of phycoerythrin (PE)-conjugated mouse anti-human CD69 (Becton Dickinson, Oxford, U.K.) or 10 µl of PE-conjugated mouse anti-human CD11b (Serotec, Oxford, U.K.). Samples were also stained with 2.5 µl of fluorescein isothiocyanate-conjugated mouse anti-human CD45 (Dako, High Wycombe, U.K.), to allow identification of PMN during flow-cytometric analysis (Figure 1). Appropriate PE-conjugated mouse IgG1 isotype controls (Becton Dickinson and Serotec) were used to control for non-specific binding. A 1 ml portion of Facslyse (Becton Dickinson) was added to
Aging-related changes in CD11b and CD69 expression

Figure 2 Determination of CD69 and CD11b expression in human PMN

Flow-cytometric scatter plots of anti-CD45 binding against anti-CD69 binding (upper panel) and anti-CD11b binding (lower panel) for PMN from one young subject. Whole blood was stimulated with 100 ng/ml PMA prior to staining with monoclonal antibodies. PMN were identified using a scatter plot of perpendicular light scatter against anti-CD45 binding, as shown in Figure 1. Scatter plots show the division of PMN into high and low antigen expression groups for both CD69 and CD11b. Data are shown for 10000 cells.

each sample to lyse erythrocytes and fix the remaining cells, and then samples were washed twice in PBS (200 g, 10 min) and analysed within 15 min using a flow cytometer (FACStar; Becton Dickinson Immunocytochemistry Systems, Mountain View, CA, U.S.A.).

Data analysis

Data were analysed using Lysys II software (version 1.1; BD 1992). Dot plots of anti-CD45–fluorescein isothiocyanate staining against either anti-CD11b–PE or anti-CD69–PE staining were constructed to identify PMN populations with high or low PE fluorescence (Figure 2). Median fluorescence intensity and the number of PMN in high- or low-fluorescence cell populations were calculated using 256-channel frequency histograms of PE fluorescence (either anti-CD11b or anti-CD69 binding). Fluorescence units were converted into molecules/cell by calibration with quantitative fluorescence bead standards (Dako Fluospheres). Non-specific binding was subtracted from total binding for each sample after conversion into molecules/cell.

Data within groups were found to be distributed non-parametrically, and statistical analysis was performed using the Mann Whitney U test.

RESULTS

Unstimulated cells

In unstimulated cells, CD11b was expressed constitutively at low levels (median number of molecules expressed per cell above background fluorescence: young (n = 15), 0.51 \times 10^4 [range (0.08–1.20) \times 10^4]; elderly (n = 15), 0.72 \times 10^4 [(0.21–1.60) \times 10^4]; P = 0.18). Levels of CD11b expression were increased if unstimulated samples were kept at higher temperatures, and therefore blood samples kept on ice throughout were used for the baseline measurement of CD11b. As has been observed previously [9], there was no constitutive expression of CD69 (young (n = 21), \(-0.13 \times 10^5\) [range (\(-0.46\) to \(0.22\)) \times 10^5] molecules/cell; elderly (n = 17), \(-0.19 \times 10^5\) \((-0.43\) to 0.05) \times 10^5] molecules/cell; P = 0.28).

Stimulation of CD11b expression with PMA

Stimulation of PMN with low PMA concentrations (1–10 ng/ml) resulted in a rapid and uniform increase in CD11b expression in all cells, which reached a maximum within 5 min, and was maintained for up to 1 h. Stimulation with higher PMA concentrations (10–100 ng/ml) again resulted in CD11b expression in all cells within the first 5 min. However, after this, a proportion of cells lost CD11b expression completely, such that expression of CD11b was lower even than in unstimulated samples (Table 1; P = 0.0001 compared with unstimulated samples), and started to express CD69. Cells that retained CD11b expression continued to express CD11b at maximal levels for up to 1 h. The PMA concentration curves (Figure 3) and time courses for CD11b expression were similar for young and elderly subjects.

Following incubation with PMA (100 ng/ml) for 30 min, no differences between young and elderly subjects were observed in either the percentage of cells that retained CD11b expression or the maximal levels of CD11b expressed per cell (Table 1).

Stimulation of CD69 expression with PMA

Stimulation of PMN with low PMA concentrations (1–10 ng/ml) for up to 1 h resulted in minimal CD69 expression. At higher PMA concentrations (10–100 ng/ml), stimulation resulted in a gradual increase in the proportion of PMN expressing CD69 at a high level, reaching a plateau at a PMA concentration of 20 ng/ml (Figure 3). As cells expressed CD69, they lost granularity, suggesting that a shape change had occurred [11], and showed reduced expression of the common leucocyte marker CD45 (Figure 2). Cells that expressed CD69 at high levels did not express any CD11b, and those that
Table 1  CD11b and CD69 expression following stimulation with PMA or fMLP
Expression of CD11b and CD69 was measured on human PMN following stimulation with 100 ng/ml PMA for 30 min or with 0.1 μM fMLP for 15 min. Results show the median (range) values for the percentage of cells within the high antigen expression group for CD69 or CD11b, and the number of molecules expressed per cell in the high and low antigen expression groups. A significant decrease in the percentage of cells expressing CD69, but not of those expressing CD11b, was observed following stimulation with PMA. There was no change in the number of molecules of CD69 or CD11b expressed per cell in either the high or low antigen expression groups following stimulation with PMA or fMLP. Non-specific binding values were subtracted. The significance of differences (P) between the young and elderly groups were tested using the Mann–Whitney U test.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Elderly</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells in high-expression group (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA-stimulated CD69 expression</td>
<td>55.3 (33.8–81.1) (n = 21)</td>
<td>43.9 (25.9–65.8) (n = 17)</td>
<td>0.01</td>
</tr>
<tr>
<td>PMA-stimulated CD11b expression</td>
<td>73.7 (49.8–97.2) (n = 13)</td>
<td>68.4 (44.6–83.6) (n = 14)</td>
<td>0.15</td>
</tr>
<tr>
<td>fMLP-stimulated CD11b expression</td>
<td>100 (n = 15)</td>
<td>100 (n = 15)</td>
<td></td>
</tr>
<tr>
<td><strong>10^3 × Molecules/cell</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA-stimulated CD69 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-expression group</td>
<td>7.10 (2.1–23.0)</td>
<td>8.31 (4.3–13.6)</td>
<td>0.66</td>
</tr>
<tr>
<td>Low-expression group</td>
<td>0.37 (0.04–0.7)</td>
<td>0.26 (0.06–0.8)</td>
<td>0.43</td>
</tr>
<tr>
<td>PMA-stimulated CD11b expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-expression group</td>
<td>12.5 (6.7–26.0)</td>
<td>12.7 (3.3–50.2)</td>
<td>0.87</td>
</tr>
<tr>
<td>Low-expression group</td>
<td>−0.15 (−0.6 to 0.4)</td>
<td>−0.21 (−0.6 to 0.7)</td>
<td>0.34</td>
</tr>
<tr>
<td>fMLP-stimulated CD11b expression</td>
<td>13.0 (6.5–22.0)</td>
<td>13.2 (8.2–43.8)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

No differences in the median number of CD69 molecules expressed per cell in either the high or low antigen expression groups were observed between the young and elderly groups (Table 1).

**Stimulation with fMLP**
On stimulation with fMLP, CD11b binding increased rapidly on all cells, reaching a plateau after 10 min, which was maintained for up to 30 min. No differences in the time course of CD11b expression following fMLP stimulation, or in the total amount of CD11b expressed per cell following stimulation with fMLP for 15 min (Table 1), were observed between the young and elderly groups. CD69 expression was only minimally increased by fMLP stimulation (median no. of molecules per cell: young (n = 12), 0.22 × 10^3 [range (0–0.4) × 10^3]; elderly (n = 7), 0.26 × 10^3 [(0–0.5) × 10^3]; P = 0.70).

**DISCUSSION**
**Effect of subject age on surface expression of CD11b and CD69 in human PMN**
In human PMN, increasing subject age was associated with a reduction in the proportion of cells able to express CD69 following stimulation with PMA, but there was no change in the expression of CD11b after stimulation with either fMLP or PMA. The origin of this aging-related change could be either at the stage of PKC activation, where an aging-related decline in PKC function may...
impaired CD69 expression but not affect the more readily expressed CD11b, or distal to PKC activation, in the process that results in CD69-containing vesicles moving towards and fusing with the plasma membrane. Aging-related alterations in PKC activity and translocation have been reported previously [12,13], and could contribute to the observed change. The finding that there was no aging-related change in the number of molecules of CD11b or CD69 expressed following stimulation with fMLP or PMA suggests that the total amount of CD11b and CD69 per cell is unchanged with age.

CD69 expression has been reported previously as being reduced with increasing subject age in resting and stimulated T lymphocytes [14–16]. However, in this cell type CD69 expression is dependent on new protein synthesis rather than on mobilization of previously formed CD69 from intracellular vesicles. Previous studies of CD11b expression in isolated human PMN have shown decreased [17] or unchanged [18] fMLP-stimulated CD11b expression in older subjects compared with younger ones, while one study found no change in CD11b expression in unstimulated isolated monocytes [19]. However, the results of these studies may not be directly comparable with ours, as cell isolation may cause cell activation [20].

Effects of stimulation on CD11b and CD69 expression
fMLP and PMA both activate PKC, but whereas PMA binds directly to it, promoting the formation of the highly active but short-lived product PKM, fMLP activates PKC indirectly, via a cell surface receptor and inositol phosphate hydrolysis [3,21]. Prolonged stimulation of PMN with PMA results in the eventual down-regulation of PKC [21]; however, the fact that cell surface levels of CD11b and CD69 did not decrease during up to 1 h of stimulation with PMA suggests that PKC down-regulation did not affect the results of the present study. Using flow cytometry, a technique that allows the determination of antigen expression on individual cells, we observed that cells do not express CD11b and CD69 concurrently, and that, whereas CD11b is readily expressed following stimulation with fMLP or with low PMA concentrations, higher concentrations of PMA are required for CD69 expression. Higher PMA concentrations seem to result in a fundamental change in intracellular functioning, which is associated with PMN shape change [11], complete removal of CD11b and reduced CD45 expression on the cell surface, such that cells are now able to express CD69. The mechanisms underlying the switch from CD11b to CD69 expression are unclear. The clear difference in the effects of low and higher PMA concentrations suggests that different PMA concentrations have different effects upon the cell, either by differential activation of PKC or by acting on different PKC isoforms [22,23]. An alternative hypothesis is that higher PMA concentrations activate intracellular enzymes other than PKC, as it is now clear that PKC is not the only substrate for PMA [24–26].

Effects of PMN stimulation on cell shape, antigen distribution and PMN aggregation
Stimulation of PMN causes them to change shape, for example by membrane ruffling and the production of pseudopodia. Stimulation of PMN with 100 ng/ml PMA has been reported to cause PMN aggregation. However, this did not affect the results of the present study, since light microscopy of cell suspensions did not reveal evidence of cell aggregation. In addition, the forward light scatter of a cell travelling through the flow cytometer is directly related to the square of its radius [27]. If aggregation occurred, an increase in forward light scatter would be expected, which was not observed in the present study (results not shown). Furthermore, the PMN density used in incubations with 100 ng/ml PMA was below the threshold PMN density at which aggregation can occur [28]. Thus PMA-stimulated PMN aggregation did not occur in the present study.

Previous work has shown that CD11b is uniformly distributed over the cell surface in unstimulated PMN, but increases in amount and becomes localized on cell protrusions following cell stimulation [29]. There is no evidence that this altered distribution of antigen affects the fluorescence reading obtained from flow cytometry, which measures total fluorescence of individual cells. Cycling of antigens such as CD11b between the plasma membrane and intracellular compartments, via clathrin-mediated vesicle endocytosis and subsequent exocytosis, has been reported previously [30]. However, our finding that CD11b and CD69 levels on the cell surface did not decrease for up to 1 h suggests that antigen cycling did not occur during the relatively short periods over which we assessed cell function.

PMN heterogeneity
It was not possible to induce all cells to express CD69 in either subject group, even at saturating PMA concentrations (> 20 ng/ml), confirming previous observations that neutrophil populations are heterogeneous [31]. We have shown that, rather than a global reduced responsiveness within all cells, aging is associated with an increase in the proportion of non-reacting cells within a cell population, i.e. an increase in heterogeneity. This may be an important distinction to make in aging research. Much previous aging research has been performed with either cell suspensions, or whole tissues or organs, making it impossible to determine whether an age-associated reduction in function is due to a decline in function in all cells or to an increase in heterogeneity within the cell population. However, increases in het-
erogeneity have been reported with increasing age both in human T and B lymphocyte responsiveness [32] and in the accumulation of cytochrome c oxidase negative fibres in human skeletal muscle [33], suggesting that alterations in heterogeneity within cell populations may be widespread and important in the pathophysiology of aging.

In conclusion, aging is associated with an impairment in the insertion of CD69-containing vesicles into the plasma membrane of human PMN. The aging-related decrease in CD69 expression is not compensated for by an increase in the number of circulating PMN in elderly subjects [34]. Thus this impairment in the externalization of CD69-containing vesicles is likely to be related to the impairments in PMN phagocytosis, bactericidal activity and release of reactive oxygen species [7] seen with increasing age. Such changes contribute to the age-associated decrease in cellular immunity, which may predispose older people to infectious disease and tumours [35–37]. Alterations in vesicle insertion in other cell types could account for the decline in insulin sensitivity [38,39] and hormone secretion [40–42] seen in older individuals. The present study therefore provides insight into some of the mechanisms underlying intracellular signal transduction that may contribute to the physiological changes seen in human aging.

ACKNOWLEDGMENTS

J. M. Noble and others

REFERENCES

14 Rea, I. M., McNerlan, S. E. and Alexander, H. D. (1999) CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-a, IFN-g, and sIL-2R levels in aging. Exp. Gerontol. 34, 79–93
Aging-related changes in CD11b and CD69 expression


Received 1 February 1999/27 April 1999; accepted 3 June 1999