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Selective up-regulation of human granulocyte integrins and complement receptor 1 by cytokines

G. A. LIMB, A. S. HAMBLIN, R. A. WOLSTENCROFT & D. C. DUMONDE *Immunology Research Unit, The Rayne Institute, St Thomas' Hospital, London*

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SUMMARY

The percentage of human granulocytes expressing the integrins CD11b and CD11c as well as complement receptor 1 (CD35) was increased by short-term incubation of whole blood with interleukin-2 (IL-2), interleukin-4 (IL-4) and tumour necrosis factors alpha and beta (TNF-α and TNF-β). The mean fluorescence intensity of granulocyte CD18 was also increased by the above cytokines, whilst that of CD11b was only increased by TNF-α. Up-regulation of granulocyte CD18 expression was seen with 1 U/ml of IL-2, TNF-α or TNF-β, in contrast to the effect of IL-4 which was only observed with 100 U/ml. Similarly, enhanced expression of CD35 was induced by 1 U/ml of IL-2 or TNF-α but not by concentrations of IL-4 or TNF-β lower than 100 U/ml. Cytokine effects on the CD11/CD18 complex and CD35 molecules were not modified by cycloheximide, suggesting that their increased expression was not due simply to synthesis *de novo*. None of the granulocyte surface determinants investigated was altered upon short-term incubation of blood with either IL-1, IL-6 or interferon-gamma (IFN-γ). The demonstration *in vitro* that cytokines selectively up-regulate granulocyte integrins and complement receptor 1, suggests that similar mechanisms may be operating during the control of granulocyte-mediated inflammatory processes.

INTRODUCTION

Polymorphonuclear (PMN) leucocyte functions, such as adherence, transendothelial migration and phagocytosis, are initiated through cell membrane recognition and signalling.[1] An important group of adhesion molecules involved in granulocyte interaction with other cells and with antigens are the surface glycoproteins known as the CD11/CD18 antigen complex.[2] These molecules belong to the integrin family and are composed of three heterodimers with a common β subunit (CD18) non-covalently linked to each of three α-subunits, CD11a (LFA-1), CD11b (MAC-1, CR3) and CD11c (p150,95, CR4).[2] Another surface glycoprotein involved in the regulation of PMN function is the complement receptor 1 (CR1) or CD35 molecule. This receptor not only mediates clearance of immune complexes from the circulation,[3] but also immune adherence, phagocytosis and extracellular cytocidal reactions.[4]

Cytokines are potent mediators of inflammation[5] and stimulate several granulocyte functions *in vitro*. Tumour necrosis factor alpha and beta (TNF-α, TNF-β) promote adherence to nylon, inhibition of chemotactic migration and superoxide anion release by PMN.[6] Interferon-gamma (IFN-γ) enhances granulocyte Fcγ receptor expression[7] and shares with granulocyte–macrophage colony-stimulating factor (GM-CSF) the capacity to enhance PMN phagocytosis, antibody-dependent cellular cytotoxicity and production of O_2^- radicals.[7,8] In addition, granulocyte exposure to the chemotactic factor FMLP enhances cell adhesiveness and the surface expression of CD11b/CD18[9] and CD35.[10] It is thus possible that cytokine modulation of granulocyte functions may be effected via their expression of cell-surface adhesion molecules. The aim of the present study was to investigate *in vitro* the effect of cytokines on the expression of PMN CD11/CD18 integrins and CD35 molecules within unfractionated whole blood, thereby simulating the inflammatory micro-environment more closely than by the use of separated leucocytes. For this purpose, whole blood was cultured with either human recombinant interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), TNF-α and TNF-β or IFN-γ, and the expression of the integrins and of CD35 molecules on PMN leucocytes was determined by flow cytometry.

Correspondence: Dr G. A. Limb, Immunology Research Unit, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, U.K.

MATERIALS AND METHODS

Experimental design

Fresh whole blood was incubated for 3 hr in the presence or absence of human recombinant cytokines and was then fixed mildly with paraformaldehyde before red cell lysis with Tris/ammonium chloride. Leucocytes obtained in this manner were labelled with monoclonal antibodies to CD11/CD18 and CD35 and processed for flow cytometry. The composition of the

leucocyte population studied was checked by labelling aliquots of all cell samples with specific antibody to granulocytes (anti-CD15). Specificity controls for the staining of adhesion molecules comprised the use of isotype-matched monoclonal antibodies to unrelated antigens. Granulocyte surface molecule expression was recorded as percentage of positively stained cells as well as staining density (MFI ratio: see below). The effect of different cytokines on PMN was studied at corresponding unitage. To investigate whether cytokine modulation of granulocyte surface molecules involved protein synthesis or induction of TNF-α or TNF-β, cycloheximide or polyclonal anti-TNF-α or anti-TNF-β were added to blood cultures.

Blood culture and leucocyte preparation

Peripheral blood was obtained from healthy individuals and anti-coagulated with 10 U/ml preservative free heparin (Leo Laboratories Ltd, Princes Risborough, Aylesbury, Bucks). Within 5 min, aliquots (0·5 ml) of whole blood were dispensed into 5-ml polystyrene tissue culture tubes (Falcon, Becton-Dickinson, Lincoln Park, NJ) and incubated for 3 hr at 37° with graded concentrations of cytokines. After incubation, volumes (0·5 ml) of 0·4% formaldehyde (EM grade) in phosphate-buffered saline (PBS), pH 7·3, were added to the tubes, which were further incubated for 4 min at 37°. Red cells were then lysed at 37° for 5 min with 20 ml of pre-warmed 0·83% NH_4Cl in 0·01 mol/l Tris-HCl buffer, pH 7·3. The mixture was centrifuged at 300 ***g*** for 5 min, the cell pellet washed twice with cold PBS and the leucocytes finally resuspended in Tris-HCl buffer at a concentration of 1×10^7/ml.

Human recombinant cytokines

IL-1β was kindly supplied by Dr N. Smithers (Glaxo Group Research Ltd, Greenford, Middlesex, U.K.) and had a specific activity of 1×10^6 U/mg on the murine T-cell line NOB1. IL-2 was kindly provided by Ortho Pharmaceuticals (Rainton, NJ) and had a specific activity of $8{\cdot}9 \times 10^6$ U/mg on the IL-2-dependent CTLL line. IL-4 was donated by Dr K. Arai (DNAX Laboratory, Palo Alto, CA) as a supernatant from Cos 7 cells transfected with a pcD rector containing the human IL-4 cDNA clone, with its biological activity determined by anti-IgM B cell co-stimulatory assay. IL-6 was a kind gift of Dr W. Sebald (Institute of Physiology, Warzburgh, Germany) and had a specific activity of 2×10^8 U/mg on the IL-6 dependent $7TD_1$ cell line. IFN-γ was kindly supplied by Dr A. Morris (Warwick University, U.K.) and had a specific activity of 3×10^6 anti-viral U/mg on WISH cells. TNF-α was obtained from British Biotechnology (Oxford, Oxon, U.K.) and had a specific activity of 2×10^7 U/mg on L929 cells. TNF-β was supplied by the National Institute for Biological Standards and Control (NIBSC, South Mimms, Herts, U.K.) and had a specific activity of $1{\cdot}5 \times 10^8$ U/mg on L929 cells.

Flow cytometric (FACS) analysis of granulocytes

The PMN surface expression of CD11a, CD11b, CD11c, CD18 and CD35 molecules was determined by flow cytometry in a FACScan (Beckton-Dickinson, Mountain View, CA). Using round-bottomed flexible microtitre plates (Falcon, Beckton-Dickinson) leucocytes (25-μl volumes) were incubated on ice for 30 min with equal volumes of optimal dilutions of monoclonal antibody (mAb) MHM 24, detecting CD11a, mAb MHM 23 detecting CD18 (kind gift of Professor A. McMichael, John Radcliffe Hospital, Oxford, U.K.), mAb 44 detecting CD11b, mAb 3.9 detecting CD11c, mAb E11 detecting CD35, mAb 29 detecting CD15 (kind gifts of Dr N. Hogg, ICRF, London, U.K.) and mAb UCHT1 and UCHMI, detecting CD3 and CD14, respectively (kind gifts of Dr P. Beverley, University College and Middlesex Hospital Medical School, London, U.K.). Cells were washed twice with cold RPMI-1640 culture medium (Gibco) containing 2 mmol/l L-glutamine (Gibco), 100 U/ml penicillin (Glaxo, Greenford, Middlesex, U.K.), 40 μg/ml gentamicin (Rousell Laboratories, Dublin, Ireland) and 5% heat-inactivated foetal calf serum (FCS). Fluorescein isothiocyanate (FITC)-labelled $F(ab)_2$ fragment of rabbit antimouse antibody (25 μl) (Dakopatts, Glostrup, Denmark) was then added and the cells were incubated for a further 30 min on ice. Finally, leucocytes were washed twice with cold RPMI-1640 medium containing 5% FCS and resuspended in 300 μl of 1% paraformaldehyde in PBS for FACS analysis. Non-specific background fluorescence was determined by using two murine mAb, PDS-1 (IgG1) and BDS-4 (IgG2a), raised against retinal S-antigen[10] and which do not react with human leucocytes; as well as a FITC-labelled $F(ab)_2$ preparation of rabbit anti-mouse immunoglobulins. Using forward and side-scatter dot plot, a gate was set on PMN which excluded monocytes and lymphocytes. The percentage of positive cells and their mean fluorescence intensity (MFI) (linear conversion of $\log_{10}$ of fluorescence) were determined by Consort 30 analysis of gated data. The density of expression of surface molecules was expressed as the MFI ratio, by means of the following formula:

$$\text{MFI ratio} = \frac{\text{MFI of cells treated with test mAb}}{\text{MFI of cells treated with control mAb}}.$$

Incubation of whole blood with cycloheximide and cytokines

Freshly obtained blood (0·5 ml) was cultured with 50 pmol cycloheximide (Sigma, St Louis, MO) for 3 hr at 37° either in the presence or absence of 100 U/ml of IL-2, IL-4, TNF-α or TNF-β. After the incubation, the blood was fixed with 0·5% formaldehyde and the leucocytes were prepared as indicated above.

Incubation of whole blood with anti-TNF-α antibody and cytokines

Freshly obtained blood (0·5 ml) was cultured at 37° for 3 hr with or without cytokines (100 U/ml) and either in the presence or absence of 100 neutralizing units (NU) of goat polyclonal antibodies to TNF-α or TNF-β (National Institute for Biological Standards and Control, South Mimms, Herts, U.K.). One NU was equivalent to the dilution of antiserum which reduces the titre of TNF-α or TNF-β from 10 U to 1 U/ml in standard L929 cytotoxicity assays. After incubation, the blood was fixed and the leucocytes were separated and labelled as indicated above.

Statistical analysis

The percentage of granulocytes staining positively with any given mAb, together with the corresponding MFI ratios, was determined for a group of experiments using blood samples from two to seven healthy subjects. Results were expressed as means $\pm$ SEM. Student's *t*-test was used to determine the significance of differences between corresponding results from

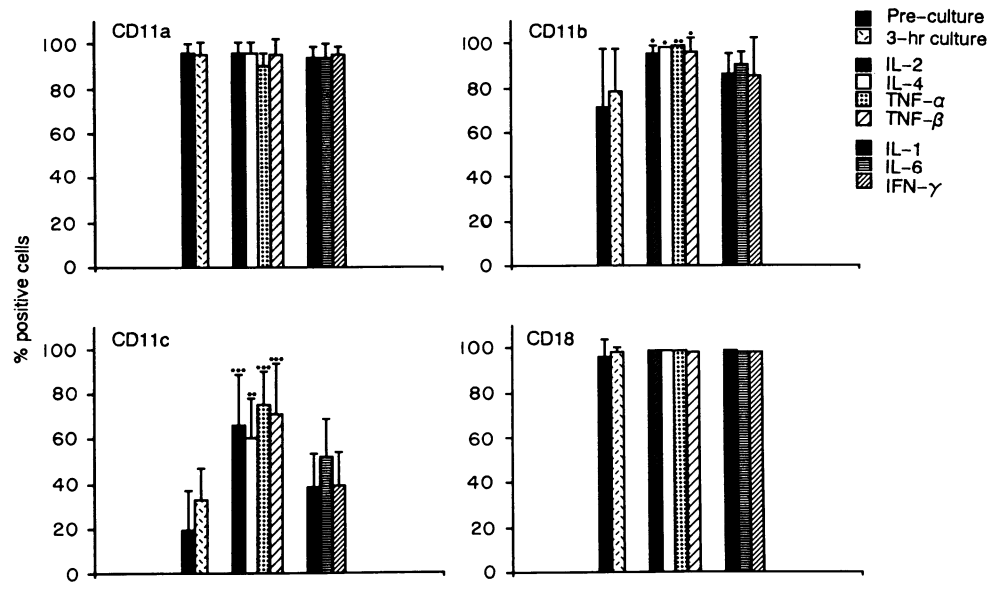

Figure 1. Cytokine effect on the percentage of PMN expressing the CD11/CD18 family of adhesion molecules. The values represent the means ± SEM of at least seven experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ (all versus 3-hr culture control). All cytokines were used at a concentration of 100 U/ml.

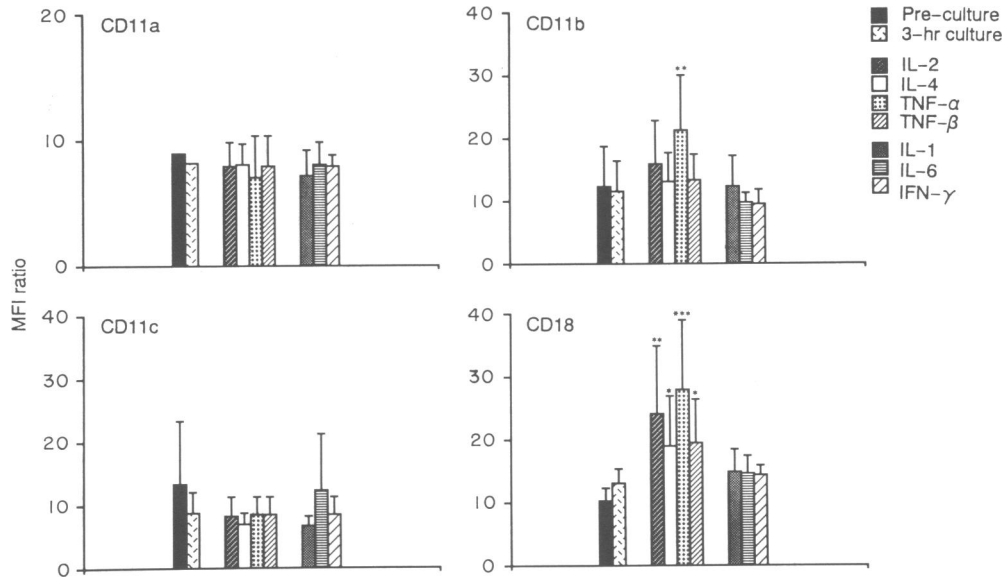

Figure 2. Cytokine effect on the density of expression of the CD11/CD18 family of adhesion molecules by PMN. The values represent the means ± SEM of at least seven experiments. $*P < 0.05$; $**P < 0.02$; $***P < 0.005$ (all versus 3-hr culture control). All cytokines were used at a concentration of 100 U/ml.

cytokine-treated and untreated cell samples. Acceptable significance was attached to differences where $P < 0.05$.

RESULTS

Effect of cytokines on the percentage of PMN bearing the CD11/CD18 molecules

Figure 1 shows the effect of different cytokines on the percentage of PMN expressing the CD11/CD18 molecules. The percentage of CD11b bearing PMN was enhanced by IL-2 ($P < 0.05$), IL-4 ($P < 0.05$), TNF-α ($P < 0.01$) and TNF-β ($P < 0.05$). Similarly, the number of PMN expressing the CD11c was significantly increased by IL-2 ($P < 0.005$), IL-4 ($P < 0.01$), TNF-α ($P < 0.005$) and TNF-β ($P < 0.005$). IL-1β, IL-6 and IFN-γ did not modify the percentage of PMN expressing CD11b or CD11c, and none of the cytokines investigated had any effect on the number of granulocytes expressing CD11a or CD18. This is not surprising as the original percentages were near the maximum.

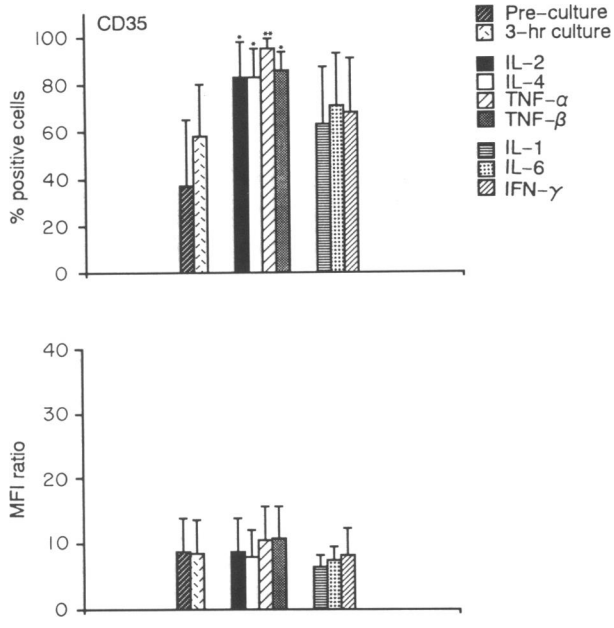

Figure 3. Cytokine effect on the expression of CD35 receptors by PMN. The values represent the means ± SEM of at least seven experiments. $*P < 0.02$; $**P < 0.001$ (both versus 3-hr culture control). All cytokines were used at a concentration of 100 U/ml.

Table 1. Effect of cycloheximide on the modulation of PMN CD18 and CD35 molecules by cytokines

	3-hr culture control	CK alone	CK + CX	CX alone
CD18 (MFI ratio)				
IL-2	14 ± 1	26 ± 8	27 ± 8	13 ± 1
IL-4	14 ± 1	21 ± 3·0	21 ± 2	14 ± 2
TNFα	15 ± 1	25 ± 1·0	25 ± 1	14 ± 1
TNFβ	15 ± 1	21 ± 1	20 ± 1	15 ± 1
CD35 (% positive cells)				
IL-2	60 ± 13	89 ± 9	91 ± 8	59 ± 9
IL-4	60 ± 13	81 ± 9	82 ± 4	58 ± 8
TNFα	57 ± 16	96 ± 3	95 ± 2	53 ± 13
TNFβ	57 ± 16	81 ± 8	81 ± 5	53 ± 12

The values represent the mean ± SEM of two different experiments.

CK, cytokine (100 U/ml); CX, cycloheximide (100 pmol/ml).

Effect of cytokines on the density of expression of CD11/CD18 molecules on PMN

Figure 2 shows the effect of different cytokines on the intensity of expression (MFI ratio) of CD11/CD18 molecules on PMN.

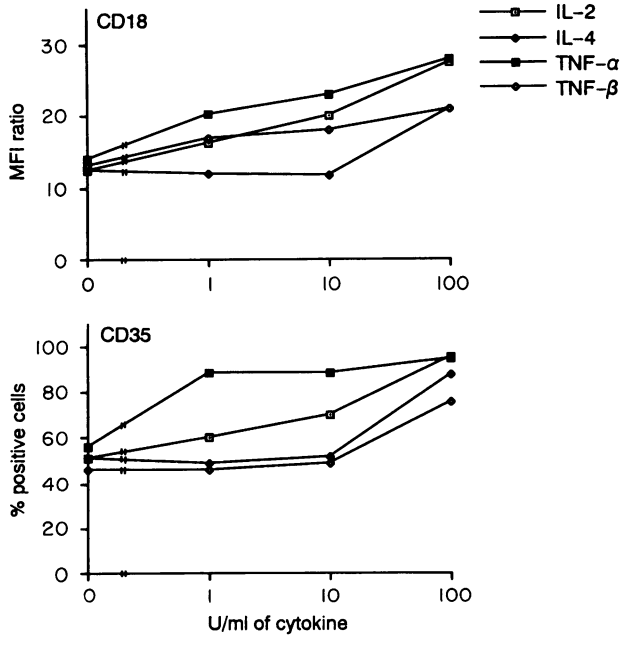

Figure 4. Effect of different concentrations of cytokines on the expression of CD18 and CD35 molecules by PMN. Representative experiment showing dose-response curves after a 3-hr activation with cytokines.

The MFI ratio for CD18 was enhanced by IL-2 ($P < 0.02$), IL-4 ($P < 0.05$), TNF-α ($P < 0.005$) and TNF-β ($P < 0.05$), but not by IL-1β, IL-6 or IFN-γ. The MFI ratio for CD11b expression on PMN was only increased by TNF-α ($P < 0.02$), and none of the cytokines investigated modified the density of expression of CD11a or CD11c.

Alteration of CD35 expression on PMN by cytokines

As seen in Fig. 3, the percentage of granulocytes expressing the CD35 molecule was markedly enhanced by IL-2 ($P < 0.02$), IL-4 ($P < 0.02$), TNF-α ($P < 0.001$) and TNF-β ($P < 0.02$). Similar to that seen with CD11/CD18, the expression of CD35 was not modified by incubation of the blood with IL-1, IL-6 or IFN-γ. None of the cytokines investigated modified the MFI ratio for this molecule on PMN leucocytes.

Effect of different concentrations of cytokines on the expression of CD18 and CD35 molecules on PMN

The maximal increase in the MFI ratio for CD18 was observed after incubation of blood with 100 U/ml of IL-2, IL-4, TNF-α or TNF-β (Fig. 4). Decreasing the concentration of IL-2, TNF-α and TNF-β reduced the level of expression of CD18. The effect of IL-2, TNF-α and TNF-β was still observed at concentrations as low as 1 U/ml. Concentrations of IL-4 below 100 U/ml did not modify the expression of CD18 molecules.

The maximum proportion of PMN expressing CD35 was observed after incubating blood with 100 U/ml of either IL-2, IL-4, TNF-α or TNF-β. Decreasing the concentrations of IL-2 diminished the percentage of granulocytes expressing CD35, although an effect was still observed with 1 U/ml of this cytokine. A reduction in the concentration of TNF-α did not cause major changes in the percentage of PMN expressing CD35; values close to the maximum were observed with 1 U/ml of this cytokine. Neither IL-4 or TNF-β modified the number of CD35 positive PMN when used at concentrations below 100 U/ml.

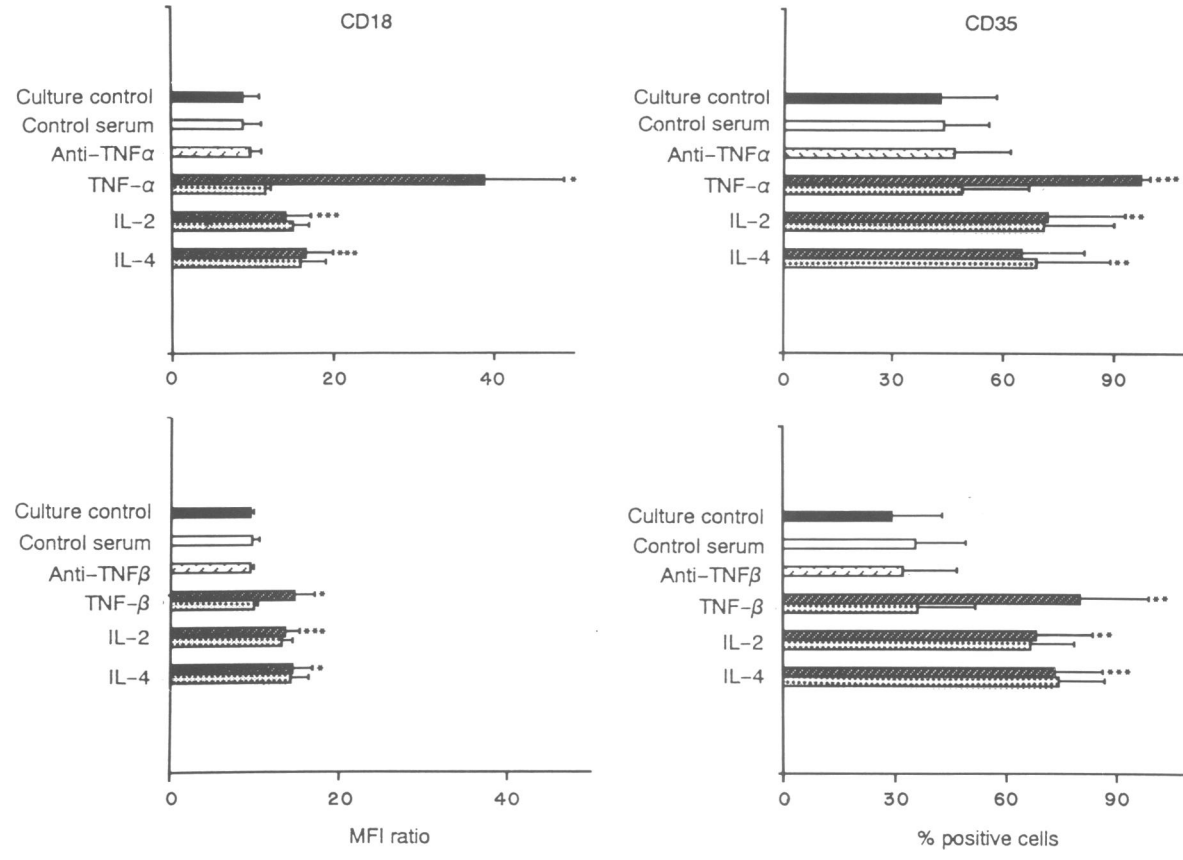

Figure 5. Effect of anti-TNF antibodies on the expression of PMN CD18 and CD35 molecules upon activation with IL-2, IL-4 and TNF-α/TNF-β. The results are the mean ± SEM of three different experiments. The heavy cross-hatch blocks represent the response of the cells to cytokines. The stippled blocks represent the response of the cells cultured with cytokine and anti-TNF-α or anti-TNF-β antibodies. $*P < 0.05$; $**P < 0.03$; $***P < 0.02$ (all versus 3-hr culture control). Normal goat serum was used as a control. All cytokines were used at a concentration of 100 U/ml.

Effect of cycloheximide on cytokine up-regulation of CD18 and CD35 expression on PMN

The level of expression of CD18 molecules on PMN and the percentage of CD35 positive granulocytes were not changed by culturing the cells in the presence of cycloheximide for 3 hr when compared with controls (Table 1). Moreover, cycloheximide did not modify the effect of IL-2, IL-4, TNF-α or TNF-β on CD18 expression on PMN or the number of PMN expressing CD35.

Effect of anti-TNF antibodies on IL-2, IL-4 and TNF-α/TNF-β modulation of CD18 and CD35 molecules on PMN

As seen in Fig. 5, the effect of both IL-2 and IL-4 on the expression of CD18 and CD35 on PMN was not altered by culturing the cells in the presence of anti-TNF-α or anti-TNF-β antibodies. In contrast, the effects of TNF-α and TNF-β were completely abrogated by their respective antibodies. These findings suggest that the effect of IL-2 and IL-4 on the modulation of CD18 and CD35 expression is not due to induction of TNF-α or TNF-β by leucocytes.

DISCUSSION

The present investigation shows that in the presence of cytokines there is a selective and rapid modulation in the expression of granulocyte surface molecules in whole blood. Granulocyte expression of CD11b, CD11c, CD18 and CD35 molecules was enhanced following short-term incubation of blood with IL-2, IL-4, TNF-α and TNF-β, in contrast to IL-1, IL-6 and IFN-γ which did not modify the expression of these molecules under the same conditions. Since blood leucocyte separation procedures, such as density gradient centrifugation[11] and cooling and rewarming,[12] increase integrin and CD35 molecule expression on neutrophils, we used whole heparinized blood incubation followed by light fixation and ammonium chloride red cell lysis to obtain the leucocytes. This simple method prevents artefactual up-regulation of cell-surface molecule expression due to cell manipulation prior to their activation and analysis (A. S. Hamblin *et al.*, unpublished observations).

The lack of cytokine effect on the percentage of PMN expressing CD11a or CD18 is not surprising since, in agreement with other published work,[13,14] most granulocytes express these two molecules. The finding that cytokines enhanced the percentage of granulocytes bearing CD11b and CD11c, but did not

significantly alter the mean intensity of the cells, may be explained as more cells expressing antigen with the same density. The reason for the increased density of expression of CD18 but not of CD11b or CD11c is not completely clear. However, it appears that very small changes in CD11b and CD11c, which are not significant on their own, when added can be seen as a more pronounced and significant effect on CD18 fluorescence intensity. Up-regulation of the expression of integrins and CD35 molecules on PMN was not due to *de novo* synthesis, as the cytokine effects were not modified by cycloheximide. This suggests that activation of PMN by cytokines may result in a redistribution of surface molecules from the intracellular pool onto the cell membrane, which is in accordance with previous reports that CD11/CD18 and CD35 molecules, normally stored within PMN granules,[15,16] are transported to the cell membrane upon activation with chemotactic agents such as FMLP.[17]

The present observations that IL-2 and IL-4 increased granulocyte expression of CD11/CD18 and CD35 molecules and the recent report that IL-2 enhances the oxidative activity and induces migration of murine PMN *in vivo*[18] suggest that these cytokines may have a direct effect on PMN. Granulocytes are not known to possess receptors for IL-2 or IL-4. However, the presence of cytokine receptors does not appear to be a precondition for PMN activation, as granulocytes, which are not known to express IFN-γ receptors, are activated by recombinant IFN-γ.[19] Since IL-2 can induce the release of TNF-α and TNF-β by mononuclear cells,[20] it was possible that such an indirect mechanism accounted for granulocyte activation. However, antibodies to TNF-α or TNF-β did not abrogate the effect of IL-2. Moreover, these antibodies did not block the effect of IL-4. These results therefore reinforce the view that IL-2 and IL-4 have a direct action on PMN.

That TNF-α enhances CD11b, CD11c, CD18 and CD35 expression on PMN is also in accordance with a recent study in which TNF-α increased granulocyte surface expression of CD11b/CD18 molecules.[21] It was not surprising that TNF-β also enhanced the expression of adhesion molecules and CD35 receptors on PMN, since both TNF-α and TNF-β share the same receptors.[22] In addition, these two cytokines modulate granulocyte adherence and superoxide anion release,[23,24] which are functions known to be mediated by the CD11b/CD18 complex.[25] IFN-γ is known to up-regulate Fcγ receptors and to enhance granulocyte functions associated with these receptors. Hence, it is conceivable that this cytokine might modify the expression of granulocyte adhesion and CD35 molecules, but that was not observed in this study, possibly due to the short time of incubation used.

The present findings, that cytokines selectively increase granulocyte surface molecules in the presence of all other formed and fluid elements of the blood, suggest that selective actions of cytokines may represent important mechanisms by which PMN functions are controlled during development and resolution of inflammatory processes.

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