Immunocomplexes stimulate different signalling events to chemoattractants in the neutrophil and regulate L-selectin and β_2-integrin expression differently

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Neutrophils express receptors for numerous phlogistons which, when occupied, trigger distinct signal-transduction pathways. Previous studies have shown that stimulation of neutrophils with chemoattractants induces shedding of the adhesive molecule L-selectin and increased expression of the β_2-integrin CD11b/CD18. We determined the effect of ligation of classic, G-protein-linked chemoattractant receptors [C5a, interleukin-8 (IL-8), formylmethionyl-leucylphenylalanine (FMLP) and substance P], receptors for the Fc portion of IgG (Fcγ receptors) and receptors for transforming growth factor β (TGFβ) on expression of adhesive molecules by neutrophils and the stimulus-transduction mechanisms thought to mediate these changes. We were surprised to observe that occupancy of Fcγ receptors by immunocomplexes (BSA–anti-BSA) stimulated increased expression by neutrophils of CD11b/CD18 at concentrations which did not affect L-selectin expression (EC_{50} 9 μg/ml versus 350 μg/ml respectively, $P < 0.00001$, $n = 5$). In contrast, similar to previous studies, recombinant C5a, recombinant IL-8 and FMLP all stimulated increased expression of CD11b/CD18 (170–260% of basal, $P < 0.001$, $n = 5$) and shedding of L-selectin (56–75% reduction from basal, $P < 0.001$, $n = 5$) at similar concentrations and with similar potencies (EC_{50} = 2, 5, and 3 nM respectively). In contrast, neither TGFβ_1 nor, surprisingly, substance P affected expression of CD11b/CD18 or L-selectin. The regulation of expression of CD11b/CD18 or L-selectin in response to FMLP or immunocomplexes was unaffected by cytochalasin B (5 μg/ml) or the tyrosine kinase inhibitor tyrphostin-25 (25 μM). Although occupancy of both chemoattractant (FMLP) and Fcγ receptors stimulated increments in the second messenger diacylglycerol, disruption of actin microfilaments by cytochalasin B enhanced diacylglycerol generation in response to FMLP but not in response to ligation of Fcγ receptors. Moreover, both FMLP and immune aggregates provoked fluxes of intracellular Ca^{2+} concentration which differed with respect to both magnitude and kinetics and did not correlate well with regulation of adhesive-molecule expression. As upregulation of CD11b/CD18 is tightly linked to exocytosis of specific granules, these results suggest that shedding of L-selectin by activated neutrophils is not linked to exocytosis. These studies provide further evidence that receptors for chemoattractants and immunocomplexes on the neutrophil are linked to multiple signalling pathways.

INTRODUCTION

Neutrophils express a variety of receptors which, when occupied by appropriate ligands, stimulate adhesion, generation of oxygen radicals ($O_2^{-\cdot}$, H_2O_2), degranulation, chemotaxis and phagocytosis. Many of these receptors have been isolated and their fine structure determined; receptors for the chemoattractants formylmethionyl-leucylphenylalanine (FMLP), C5a, interleukin-8 (IL-8) and substance P [reviewed in Lefkowitz (1991)] are members of the seven transmembrane-spanning family of G-protein-linked receptors. Neutrophils also express two distinct receptors for the Fc portion of immunoglobulin (Fc_γ). It is now clear that ligation of Fc_γ receptors by immunocomplexes stimulates neutrophil function via different pathways to those utilized by chemoattractant receptors [cf. Melnick et al. (1988); Reibman et al. (1991a)]. Moreover, even within the family of G-protein-linked receptors there are different stimulus-transduction mechanisms, as the functional consequences of stimulation by substance P differ from those stimulated by FMLP, IL-8 or C5a (Kolasinski et al., 1992).

Upon stimulation within the microcirculation at inflamed sites neutrophils adhere to the vascular endothelium and migrate into the extravascular space. The principal molecules on the neutrophil responsible for adhesion to endothelium include the glycoprotein L-selectin and the β_2 integrin CD11b/CD18 [reviewed in Lasky (1992); Lobb (1992); Paulson (1992)]. L-selectin, a member of a family of adhesion proteins that bind to carbohydrate moieties on glycoproteins via lectin-like domains, mediates 'rolling' of neutrophils along the walls of the microvasculature and the initial contact with the endothelium. After stimulation of the neutrophil by chemoattractants, L-selectin is shed from the cell surface, presumably to permit more rapid egress of the neutrophil from the vascular bed (Kishimoto et al., 1989, 1990; Jutila et al., 1991). In contrast, CD11b/CD18 must be activated before mediating tight binding to intracellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells (Schwartz et al., 1985; Anderson et al., 1986; Altieri and Edgington, 1988; Vedder and Harlan, 1988; Philips et al., 1988; Smith et al., 1988). Surface expression of CD11b/CD18 increases after stimulation of the neutrophil. Previous studies have demon-

Abbreviations used: FMLP, formylmethionyl-leucylphenylalanine; IL-8, interleukin-8; TGFβ, transforming growth factor β; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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strated that occupancy of classic chemoattractant receptors (for FMLP, C5a and IL-8) stimulates the increased expression of CD11b/CD18 and shedding of L-selectin (Kishimoto et al., 1989, 1990; Jutila et al., 1991) but the effect of immunocomplexes, substance P or the novel chemoattractant transforming growth factor β (TGFβ) on surface expression of adhesive molecules has not been thoroughly examined.

We compared the effect of immunocomplexes, classical chemoattractants (FMLP, C5a and IL-8) and the non-classic chemoattractants TGFβ and substance P on expression of CD11b/CD18 and L-selectin and studied the signal-transduction mechanisms responsible for stimulated changes in expression of these adhesive molecules. We observed that, unlike any of the other agents studied, ligation of Fc_γ receptors by immunocomplexes did not significantly alter expression of L-selectin except at doses significantly greater than those required to increase expression of CD11b/CD18. In contrast, we found that, as previously reported, classical chemoattractants stimulate coordinate shedding of L-selectin and increased expression of CD11b/CD18. Neither TGFβ nor substance P affected surface expression of either L-selectin or CD11b/CD18. Moreover, signalling via classic chemoattractant receptors differs from that via Fc_γ receptors with respect to fluxes in intracellular Ca^{2+} concentration ($[Ca^{2+}_i]$) and phospholipid remodelling. We conclude that generation of neither of these intracellular signals correlates with changes in either adhesive molecule.

MATERIALS AND METHODS

Monoclonal antibodies

Antibodies directed against CD11b (MN41, IgG1) were obtained by subcloning of hybridomas generously supplied by Dr. Allison Eddy. We have previously shown that MN41 recognizes an epitope on the CD11b/CD18 which, although required for function (e.g. homotypic aggregation), does not represent an activation state of the integrin (Buyon et al., 1988; Philips et al., 1988). Anti-(L-selectin) (DREG-56, IgG1) was prepared as previously described (Kishimoto et al., 1990). Anti-(major histocompatibility complex-class I) (W6/32, IgG2a) framework antigen was obtained by subcloning of hybridomas obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.), fluorescein-labelled goat anti-(mouse Ig) and a non-binding isotype control (MOPC 21, IgG1) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All antibodies were diluted in PBS and used at saturating concentrations, as determined in preliminary experiments.

Reagents

Cytochalasin B, colchicine, dexamethasone, FMLP, recombinant human C5a and substance P were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human recombinant $IL\text{-}8_{77}$ was purchased from R&D Systems (Minneapolis, MN, U.S.A.) and recombinant human TGFβ-1 was a generous gift from Dr. M. Palladino (Genentech, S. San Francisco, CA, U.S.A.). The tyrosine kinase inhibitor tyrphostin-25 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). All other reagents were of the highest quality that could be obtained.

Preparation of immunocomplexes

Immunocomplexes were prepared, as previously described (Ward and Zvaifler, 1973; Goldstein et al., 1975), by mixing BSA (Sigma Chemical Co.) with rabbit polyclonal anti-BSA IgG (Cappel Laboratories, West Chester, PA, U.S.A.) in an antigen/antibody ratio of 1:5 (w/w) in order to obtain precipitating complexes at the point of antigen–antibody equivalence. Alternatively, the rabbit antibody was heated at 62 °C for 10 min to form immune aggregates.

Preparation of leucocytes for analysis by flow cytometry

Anticoagulated blood (EDTA in Becton–Dickinson Vacutainer tubes) was drawn from healthy volunteers before each experiment and was kept on ice until assayed. In some experiments cytochalasin B (5 μg/ml) or tyrphostin-25 (25 μM) were added to whole blood and incubated with cells for 30 min at 37 °C before addition of appropriate stimuli. Because standard isolation procedures stimulate changes in CD11b/CD18 and L-selectin surface expression, neutrophils were not separated from whole blood before staining and fixation. Whole blood was incubated with stimulant or PBS at 37 °C for 15 min, the cells were spun down and washed with PBS before incubation with appropriate monoclonal antibodies at room temperature for 15 min. After washing the cells were incubated with fluorescein-labelled goat anti-(mouse immunoglobulin) antibody (1:200 final dilution) in the dark at room temperature for 15 min. After lysis of the erythrocytes with fluorescence-activated cell sorter lysing solution (1X, Becton–Dickinson) for 10 min, cells were washed and fixed with 0.5 ml of 5% (v/v) paraformaldehyde (Smith et al., 1991). Fluorescence staining of the leucocytes was analysed by means of a FACScan (Becton–Dickinson, San Jose, CA, U.S.A.). Neutrophils were identified by their characteristic size and 90° forward-light-scattering characteristics.

Diacylglycerol determination

Neutrophils (7.5×10^7/ml) were suspended in Hepes buffer containing fatty acid-free BSA (0.1%), [^{3}H]arachidonate (3 μCi/ml) and [^{14}C]glycerol (7 μCi/ml) and incubated (30 min, 37 °C). This incubation time was sufficient to reach plateau, as determined in preliminary experiments. Unincorporated label was removed by washing cells twice in Hepes–BSA. Neutrophils (1.7×10^7) were incubated (5 min, 37 °C) in the presence or absence of cytochalasin B (5 μg/ml) and treated with FMLP (0.1 μM) or immunocomplexes (0.2 mg/ml) for various times. The reaction was terminated by the addition of 3.5 ml of chloroform/methanol (2:5, v/v). Samples were extracted by the Bligh and Dyer technique as previously reported (Bligh and Dyer, 1959). Samples were applied to heat-activated one-dimensional silica gel GF plates and run in hexane/ether/acetic acid (50:50:1, by vol.) (Reibman et al., 1988). Lipids were visualized by exposure to iodine vapour, scraped off the plates and radioactivity counted in a Beckman LS7000 scintillation counter in 1% (w/v) sodium thiosulphate to bleach the iodine and 10 ml of Dimiscint (National Diagnostics, Mainville, NJ, U.S.A.). Diacylglycerol was identified in comparison with standards of diolein, 1,2-distearin, 1,2-distearoyl-rac-glycerol, di-eicosenoin, triarachidonin, triolein and monolein (all 97–99% pure). Neither acyl-chain substitutions nor the presence of an ether, rather than an ester, linkage of the fatty acid groups altered the mobility of diacylglycerol. These conditions result in incorporation of label into the diacyl- rather than the 1-*O*-alkyl, 2-acyl diacylglycerol as alkali hydrolysis (0.5 M NaOH, 37 °C, 15 min) of [^{14}C]diacylglycerol isolated from neutrophils generated no labelled monoacylglycerol [buffer = 62 ± 14 c.p.m./diacylglycerol fraction, 18 ± 8 c.p.m. monoacylglycerol fraction; NaOH-treated = 2 ± 1 c.p.m./diacylglycerol fraction,

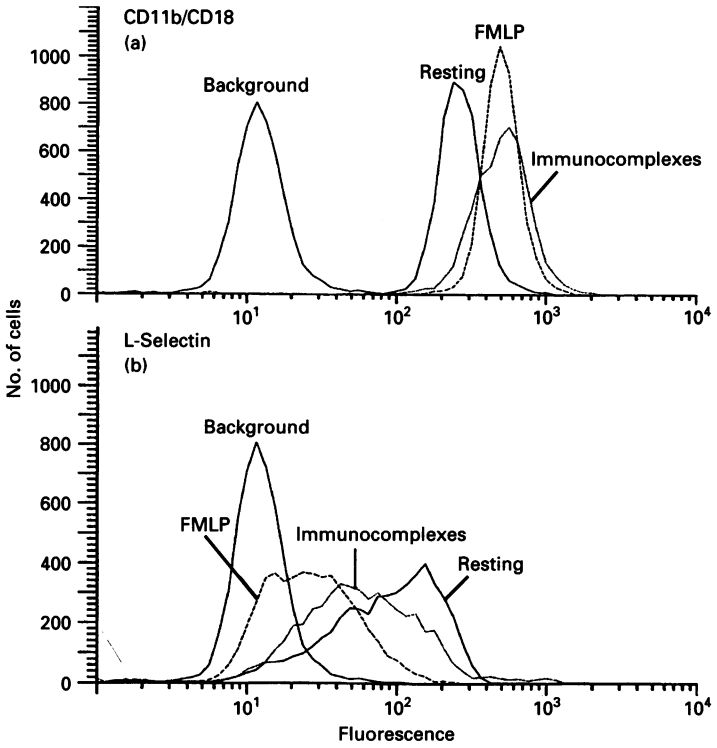

Figure 1 Unlike FMLP, immunocomplexes do not stimulate increased expression of CD11b/CD18 and loss of L-selectin in parallel

Shown are representative cytofluorograms of neutrophils incubated in whole blood in the presence of medium (control), immunocomplexes (50 μg/ml, final concentration) or the chemoattractant FMLP (100 nM). After stimulation, isolation and fixation neutrophils were incubated with mouse monoclonal antibody MN41 directed against CD11b (CD11b/CD18) or DREG-56 directed against L-selectin. In this experiment immunocomplexes stimulated 90% of the maximal increase in CD11b/CD18 expression but only 35% of the maximal decrement in L-selectin expression. Neutrophils stimulated with FMLP at this concentration increased CD11b/CD18 expression and decreased L-selectin expression maximally.

14 ± 6 c.p.m./monoacylglycerol fraction (Haines et al., 1991)]. This confirms that our assay measures solely the diacyl-phospholipid metabolites.

Measurement of $[Ca^{2+}]_i$

Neutrophils (1×10^8/ml) were incubated in 1×10^{-5} M Fura-2 AM in Hepes buffer, pH 7.4, (37 °C, 5 min). They were then diluted 10-fold with Hepes buffer, incubated for 20 min and washed twice. Preloaded cells (2 ml, 5×10^6 cells/ml) were placed in a 1 cm^2 quartz cuvette (37 °C). Fluorescence changes were monitored with an excitation wavelength of 340 and 380 nm and an emission wavelength of 510 nm in a Perkin–Elmer 6450-10S spectrofluorimeter. Heat-aggregated insoluble rabbit immunoglobulin (62 °C, 10 min) was used for these experiments as the turbidity of this preparation is sufficiently low to permit us to perform fluorescence measurements. Levels of $[Ca^{2+}]_i$ were calculated according to the equations of Grynkiewicz et al. (1985).

RESULTS

Occupancy of Fc_γ receptors induces increased expression of CD11b/CD18 without shedding of L-selectin

Exposure of leucocytes to immunocomplexes (BSA–anti-BSA) stimulated the increased expression of CD11b/CD18 by 158 ± 33%, a response comparable with that provoked by FMLP (0.1 μM, Figure 1). At concentrations which stimulated maximal increases in CD11b/CD18 expression immunocomplexes provoked only modest shedding of L-selectin, as compared with FMLP (Figure 1b). After stimulation with a maximal concentration of immunocomplexes L-selectin expression decreased by 68 ± 9%, a change which was similar in magnitude to that induced by FMLP (74 ± 2% decrease). Most importantly, the concentration of immunocomplexes required to induce changes in expression of CD11b/CD18 differed significantly from that required to modulate L-selectin ($P < 0.00001$, $n = 5$, Figure 2).

Ligation of classic chemoattractant receptors stimulates increased expression of CD11b/CD18 and L-selectin

Because of these disparate effects of immunocomplexes on surface expression of CD11b/CD18 and L-selectin we determined the effect of the classic chemoattractants (C5a and IL-8) on expression of adhesion molecules by neutrophils and compared these with changes provoked by FMLP. As previously reported (Berger et al., 1984; Todd et al., 1984; Miller et al., 1987; Kishimoto et al., 1989, 1990; Jutila et al., 1991), these three chemoattractants stimulated an increase in the surface expression of CD11b/CD18 and loss of surface expression of L-selectin (Figure 3). Maximal concentrations of FMLP, C5a and IL-8 induced increased surface expression of CD11b/CD18 by as much as 158 ± 29%, 146 ± 31% and 71 ± 23% over basal (non-stimulated neutrophil) expression respectively ($P < 0.001$ versus basal for all, $n = 5$, Figure 3). Conversely, after stimulation with the classic chemoattractants the expression of L-selectin decreased by 74 ± 2%, 72 ± 3%, and 56 ± 2% respectively ($P < 0.001$ versus basal for all, $n = 5$). The classic chemo-

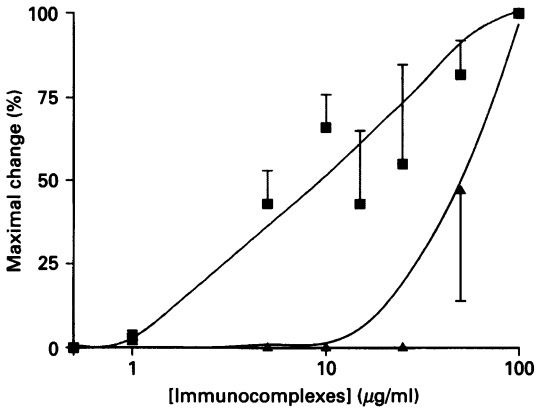

Figure 2 Immunocomplexes do not stimulate increased expression of CD11b/CD18 and loss of L-selectin in parallel

Shown are the means (±S.E.M.) of five different experiments. Analysis of variance demonstrates that immunocomplexes stimulate upregulation of CD11b/CD18 (■) at significantly lower concentrations than those required to stimulate downregulation of L-selectin (▲).

attractants regulated surface expression of CD11b/CD18 and L-selectin at similar concentrations and with similar potencies (EC_{50} 3 nM, 2 nM and 5 nM, for FMLP, C5a and IL-8 respectively, Figure 4).

Changes due to classic chemoattractants in the surface expression of CD11b/CD18 and L-selectin are unaffected by disruption of actin filaments

Previous studies have demonstrated that agents which inhibit actin-filament formation (cytochalasin B) increase O_2^- generation, release of lysosomal enzymes and homotypic adherence by activated neutrophils (Zigmond and Hirsch, 1972; Goldstein et al., 1973; Roos et al., 1976). Accordingly we determined whether disruption of the cytoskeleton by cytochalasin B affected either resting or stimulated (classic chemoattractants) surface expression of CD11b/CD18 and L-selectin. Despite the well-described effects of cytochalasin B on homotypic adhesion, O_2^- generation and degranulation cytochalasin B affected neither basal nor FMLP-stimulated expression of either CD11b/CD18 or L-selectin (Figures 4 and 5).

Both immunocomplexes and chemoattractants stimulate increments in $[Ca^{2+}]_i$

As shown above, upregulation of CD11b/CD18 and shedding of L-selectin are co-ordinately controlled after stimulation with FMLP but not with immunocomplexes. We, therefore, tested the hypothesis that distinct second messengers are required for signalling at these two different types of receptor. Increments in $[Ca^{2+}]_i$ after treatment of neutrophils with FMLP and immune aggregates were distinctly different. Occupancy of both chemoattractant and Fc_γ receptors provoked a significant increment in $[Ca^{2+}]_i$; however, both the magnitude of the rise and the time to peak $[Ca^{2+}]_i$ were distinctly different; FMLP induces a larger and more long-lasting increment in $[Ca^{2+}]_i$ (129 nm versus 51 nM, FMLP versus immune aggregates; $P < 0.001$, $n = 3$, Figure 6). Moreover, peak increments in $[Ca^{2+}]_i$ were achieved more rapidly in FMLP-treated than immune aggregate-treated cells (10 s versus 20 s respectively, Figure 6).

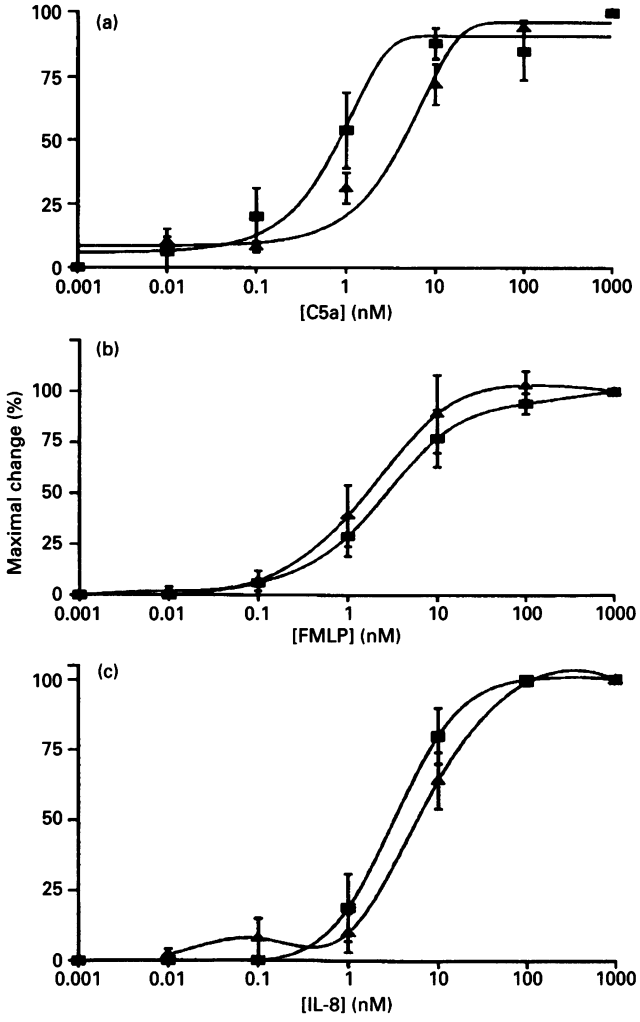

Figure 3 Chemoattractants stimulate upregulation of CD11b/CD18 (■) and downregulation of L-selectin (▲) in parallel

Shown are representative cytofluorograms of neutrophils incubated in whole blood in the presence of medium alone (control), FMLP (100 nM), C5a (100 nM) or IL-8 (100 nM). After stimulation, isolation and fixation neutrophils were incubated with mouse monoclonal antibody MN41 directed against CD11b (CD11b/CD18) or DREG-56 directed against L-selectin.

Both chemoattractants and immunocomplexes stimulate phospholipid remodelling

We have previously determined (Haines et al., 1991; K. A. Haines, unpublished work) that generation of diacylglycerol is quantitatively correlated with degranulation. Ligation of receptors for FMLP or immunocomplexes provoked generation of diacylglycerol in neutrophils (Table 1). However, cytochalasin B enhanced FMLP-stimulated generation of diacylglycerol but did not affect generation of diacylglycerol stimulated by immunocomplexes. These studies indicate that whereas cytochalasin B-insensitive generation of diacylglycerol may be required for upregulation of CD11b/CD18, it can not be sufficient for shedding of L-selectin. Moreover, as cytochalasin B does not affect shedding of L-selectin after stimulation by FMLP the pool of diacylglycerol augmented by cytochalasin treatment (cytochalasin B-sensitive) is not required for loss of L-selectin from the surface.

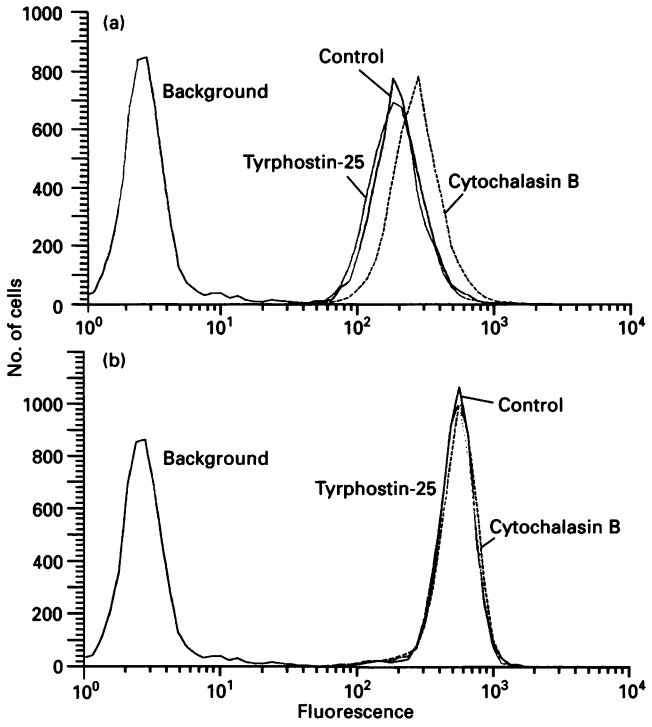

Figure 4 Neither tyrphostin-25 (25 μM) nor cytochalasin B affects resting or stimulated (FMLP, 100 nM) expression of CD11b/CD18

Shown are representative (of two different experiments) cytofluorograms of neutrophils incubated in whole blood in the presence of medium alone (control), tyrphostin-25 or cytochalasin B. After stimulation, isolation and fixation neutrophils were incubated with mouse monoclonal antibody MN41 directed against CD11b (CD11b/CD18).

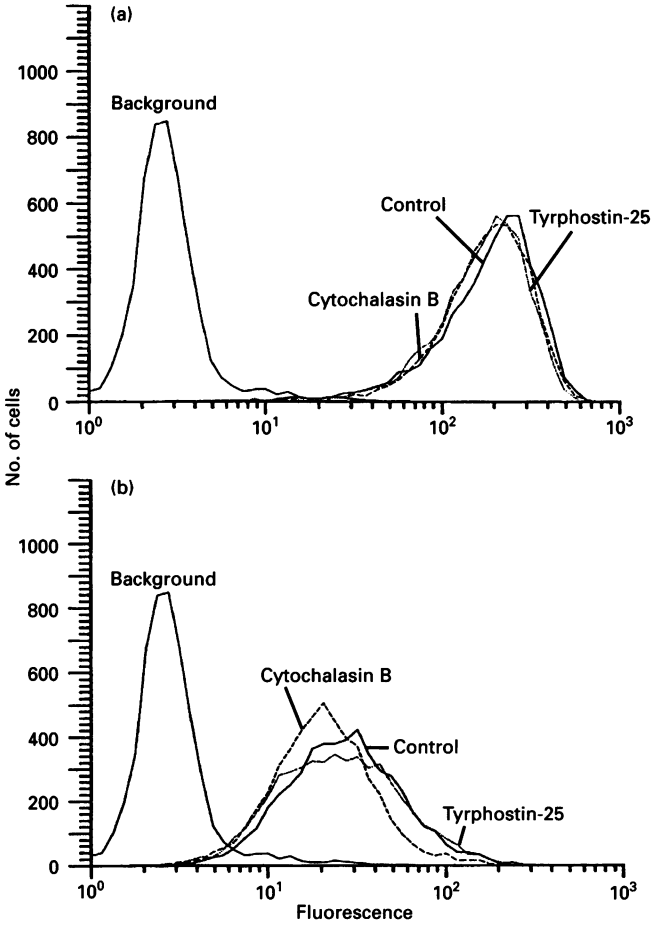

Figure 5 Neither tyrphostin-25 (25 μM) nor cytochalasin B affects resting or stimulated (FMLP, 100 nM) expression of L-selectin

Shown are representative (of two different experiments) cytofluorograms of neutrophils incubated in whole blood in the presence of 100 nM FMLP alone (control) or 100 nM FMLP plus tyrphostin-25 or cytochalasin B. After stimulation, isolation and fixation neutrophils were incubated with mouse monoclonal antibody DREG-56 directed against L-selectin.

Inhibition of tyrosine kinase activity does not affect either resting or stimulated expression of CD11b/CD18 and L-selectin

Tyrosine phosphorylation is critical for generation of O_2^- in response to classic chemoattractants (Huang et al., 1988, 1990; Gomez Cambronero et al., 1989; Nasmith et al., 1989; Berkow and Dodson, 1990; Naccache et al., 1990). Unexpectedly, the tyrosine kinase inhibitor tyrphostin-25, at a concentration which caused > 90% inhibition of stimulated O_2^- generation (25 μM, results not shown), did not affect FMLP-stimulated increases in expression of CD11b/CD18 or stimulated decreases in surface expression of L-selectin (Figures 4 and 5).

TGFβ-1 and substance P do not alter the expression of CD11b/CD18 or L-selectin on neutrophils

As classical chemoattractants regulate the surface expression of both CD11b/CD18 and L-selectin we studied the effect of optimal concentrations of two recently described chemoattractants, TGFβ-1 (1 pg/ml) and substance P (100 nM), on the surface expression of these adhesion molecules. Neither TGFβ-1 nor substance P induced any change in the surface expression of either CD11b/CD18 or L-selectin on neutrophils. Neutrophils exposed to TGFβ-1 expressed 91±4% ($n = 3$) of resting CD11b/CD18 and 107±9% of basal L-selectin ($n = 3$). Similarly, after stimulation with substance P neutrophils expressed

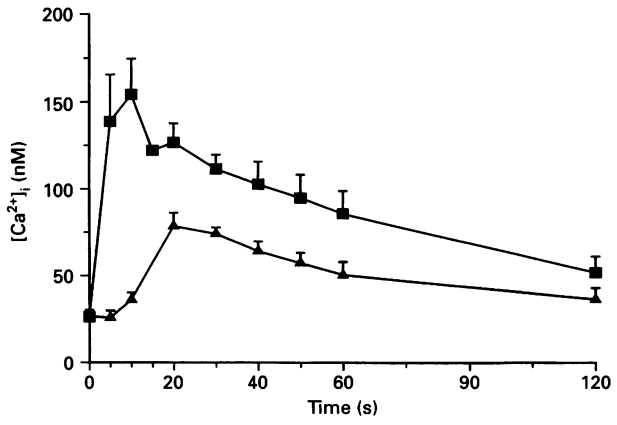

Figure 6 FMLP (100 nM; ■) and immune aggregates (0.3 mg/ml; ▲) stimulate fluxes in $[Ca^{2+}]_i$ which differ in magnitude, onset and duration

Neutrophils were preloaded with Fura-2-AM as described in the Materials and methods section. Polymorphonucleocytes (2.5×10^6) were treated at 37 °C with the above ligands and fluorescence was measured online for 2 min. Shown are the means (±S.E.M.) of three separate experiments performed in duplicate.

Table 1 Effect of cytochalasin B on [^{3}H]diacylglycerol generation

Neutrophils were labelled with [^{3}H]arachidonate as described in the Materials and methods section. Neutrophils (1.7×10^7) were incubated (5 min, 37 °C) in the presence or absence of cytochalasin B (5 μg/ml) and treated with ligands for 60 s. The reaction was terminated, lipids were separated by t.l.c. as described and diacylglycerol was scraped and counted. Resting [^{3}H]diacylglycerol S.E.M. = 2303 ± 141 (c.p.m.).

Condition	[^{3}H]Diacylglycerol generation	
	+ Cytochalasin B (% resting at 60 s)	− Cytochalasin B (% resting at 60 s)
FMLP (1×10^{-7} M)	155 ± 11	111 ± 3*
Immunocomplexes (0.2 mg/ml)	138 ± 5	141 ± 25†

* $P < 0.05$, FMLP ± cytochalasin B, $n = 3$–9 experiments.
† P = NS IC ± cytochalasin B.

92 ± 5% ($n = 3$) of basal CD11b/CD18 and 102 ± 10% ($n = 3$) of basal L-selectin.

DISCUSSION

Occupancy of receptors for classic chemoattractants stimulates increased expression of CD11b/CD18 and diminished expression of L-selectin. In contrast, immunocomplexes stimulate the increased expression of CD11b/CD18 in the absence of a concomitant loss in the surface expression of L-selectin. Previous studies have shown that a large number of chemoattractants and cytokines stimulate reciprocal changes in CD11b/CD18 and L-selectin expression [cf. Kishimoto et al. (1989)]. In general, those agents which stimulate increased expression of CD11b/CD18 also provoke shedding of L-selectin but the effects of only a single dose of the stimulants have been examined (Kishimoto et al., 1989; Griffin et al., 1990). The disparity between shedding of L-selectin and upregulation of CD11b/CD18 in response to stimulation by low concentrations but not high concentrations of immunocomplexes is striking. This observation further supports the notion that signalling at chemoattractant receptors differs from signalling at Fc receptors.

Previous studies have demonstrated that stimulus transduction at classic chemoattractant receptors differs from that at the Fc_γ receptor ($Fc_\gamma R$) [cf. (Melnick et al., 1988; Reibman et al., 1991a)]. Stimulus transduction at classic chemoattractant receptors proceeds via activation of pertussis toxin-sensitive G-proteins whereas, for the most part, stimulus transduction at $Fc_\gamma R$ proceeds via G-protein-independent pathways [reviewed in (Snyderman et al., 1986)]. It is tempting to speculate that loss of surface L-selectin results directly from activation of a pertussis toxin-sensitive G-protein. Our results do not support a direct role for the generation of phospholipid metabolites, such as diacylglycerol, in the shedding of L-selectin, as cytochalasin B enhances dramatically the increment in diacylglycerol which follows stimulation by chemoattractant but does not affect stimulated changes in L-selectin expression. Similarly, fluxes in $[Ca^{2+}]_i$ do not appear to regulate, directly, the expression of adhesion molecules since both chemoattractants and immunocomplexes stimulate Ca^{2+} fluxes. Thus, the intracellular messengers which regulate the expression of L-selectin remain to be elucidated.

Increased expression of CD11b/CD18 is tightly linked to exocytosis of specific granules, as CD11b/CD18 is mobilized to the surface of neutrophils from intracellular vesicles which co-isolate with specific granules upon subcellular fractionation (O'Shea et al., 1984; Todd III et al., 1984; Miller et al., 1987; Stevenson et al., 1987). Thus it is not surprising that occupancy of those receptors (FMLP, C5a, IL-8 and $Fc_\gamma R$) which promote secretion of specific-granule contents (e.g. lysozyme or vitamin B_{12}-binding protein) leads to increased expression of CD11b/CD18. Similarly, it is not surprising that neither substance P nor TGFβ-1, ligands that do not provoke exocytosis, do not stimulate increased expression of CD11b/CD18 (Serra et al., 1988; Reibman et al., 1991b; Kolasinski et al., 1992). In contrast, L-selectin appears to be lost from the surface of the neutrophil as a result of proteolysis by a chymotrypsin-like activity (Jutila et al., 1991); however, the steps leading to the decreased surface expression of L-selectin have not been well characterized (Jutila et al., 1991). The results reported here indicate that degranulation, which releases a variety of proteolytic enzymes, is not sufficient to diminish surface expression of L-selectin. Indeed, immunocomplexes stimulate degranulation and increased expression of CD11b/CD18 on the surface of the neutrophil at doses 100-fold less than those required to provoke loss of L-selectin.

In response to chemoattractants (e.g. FMLP, C5a and IL-8) neutrophils migrate in a directed fashion, degranulate and produce toxic oxygen metabolites but the doses of chemoattractants required to stimulate these functions maximally increase from 100 pM to 10 nM to 100 nM respectively (Weissmann et al., 1980). The observation that similar concentrations of chemoattractants provoke similar effects on upregulation of CD11b/CD18 and shedding of L-selectin, concentrations associated with exocytosis of specific granules, suggests that there is either a single signal for these changes or signalling for these alterations in plasma membrane protein expression is either identical or tightly linked. However, our observation that different concentrations of immunocomplexes stimulate upregulation of CD11b/CD18 and downregulation of L-selectin is not consistent with either a single or linked signal-transduction mechanism for these two functions.

Previous studies have demonstrated that the receptor for substance P is a member of the family of seven transmembrane spanning, G-protein-linked receptors. Indirect evidence indicates that neutrophils also possess G-protein-linked receptors for substance P as pertussis toxin diminishes the response of neutrophils to substance P (Kolasinski et al., 1992). Substance P must stimulate neutrophil function via a different G-protein (α subunit) to receptors for classic chemoattractants as it does not stimulate phospholipid metabolism, Ca^{2+} transients or alterations in expression of surface-adhesive molecules on neutrophils in a manner similar to any of the classic chemoattractants (Serra et al., 1988; Kolasinski et al., 1992).

These differing effects of classic chemoattractants and immunocomplexes on expression of adhesion molecules suggest a mechanism for the localization of neutrophils to the vascular wall during the course of immunocomplex-mediated vasculitis. L-selectin mediates adhesion of neutrophils to the vascular endothelium giving rise to 'rolling' of neutrophils along the walls of the microvasculature. Upon stimulation by soluble chemoattractants leucocytes adhere more strongly to the endothelium, then shed L-selectin and migrate out of the vasculature. However, the continued presence of the adhesive molecule L-selectin on the surface of the neutrophil after stimulation by immunocomplexes may hamper egress of neutrophils from the vasculature. Thus, the biological effect of impaired L-selectin shedding after stimulation via $Fc_\gamma R$ may be exaggeration of neutrophil adherence to the endothelium of the microvasculature. By adhering tightly to the vascular walls the immunocomplex-activated neutrophils are

more likely to injure the vascular endothelium via the secretion of active oxygen metabolites or granule contents in close proximity to the endothelium.

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REFERENCES

Altieri, D. C. and Edgington, T. S. (1988) J. Immunol. **141**, 2656–2660
Anderson, D. C., Miller, L. J., Schmalstieg, F. C., Rothlein, R. and Springer, T. A. (1986) J. Immunol. **137**, 15–27
Berger, M. J., O'Shea, J., Cross, A. S., Folks, T. M., Chused, T. M., Brown, E. J. and Frank, M. M. (1984) J. Clin. Invest. **74**, 1566–1571
Berkow, R. L. and Dodson, R. W. (1990) Blood **75**, 2445–2452
Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–919
Buyon, J. P., Abramson, S. B., Philips, M. R., Slade, S. G., Ross, G. D., Weissmann, G. and Winchester, R. J. (1988) J. Immunol. **140**, 3156–3160
Goldstein, I. M., Hoffstein, S., Gallin, J. I. and Weissmann, G. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 2916–2920
Goldstein, I. M., Roos, D., Kaplan, H. B. and Weissmann, G. (1975) J. Clin. Invest. **56**, 1155–1163
Gomez Cambronero, J., Huang, C. K., Bonak, V. A., Wang, E., Casnellie, J. E., Shiraishi, T. and Shaafi, R. I. (1989) Biochem. Biophys. Res. Commun. **162**, 1478–1485
Griffin, J. D., Spertini, O., Ernst, T. J., Belvin, M. P., Levine, H. B., Kanakura, Y. and Tedder, T. F. (1990) J. Immunol. **145**, 576–584
Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
Haines, K. A., Reibman, J., Tang, X., Blake, M. and Weissmann, G. (1991) J. Cell Biol. **114**, 433–442
Huang, C. K., Laramee, G. R. and Casnellie, J. E. (1988) Biochem. Biophys. Res. Commun. **151**, 794–801
Huang, C. K., Bonak, V., Laramee, G. R. and Casnellie, J. E. (1990) Biochem. J. **269**, 431–436
Jutila, M. A., Kishimoto, T. K. and Finken, M. (1991) Cell. Immunol. **132**, 201–214
Kishimoto, T. K., Jutila, M. A., Berg, E. L. and Butcher, E. C. (1989) Science **245**, 1238–1241

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Kishimoto, T. K., Jutila, M. A. and Butcher, E. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2244–2248
Kolasinski, S. L., Haines, K. A., Seigel, E. L., Cronstein, B. N. and Abramson, S. B. (1992) Arthritis Rheum. **35**, 369–375
Lasky, L. A. (1992) in Adhesion: Its role in Inflammatory Disease (Harlan, J. M. and Liu, D. Y., eds.), pp. 43–64, W. H. Freeman and Co., New York
Lefkowitz, R. J. (1991) Nature (London) **351**, 353–354
Lobb, R. R. (1992) in Adhesion: Its Role in Inflammatory Disease (Harlan, J. M. and Liu, D. Y., eds.), pp. 1–18, W. H. Freeman Co., New York
Melnick, D. A., Noriega, F., Brunckhorst, B. and Meshulam, T. (1988) Clin. Res. **36**, 464a
Miller, L. J., Bainton, D. F., Borregaard, N. and Springer, T. A. (1987) J. Clin. Invest. **80**, 535–544
Naccache, P. H., Gilbert, C., Caon, A. C., Gaudry, M., Huang, C. K., Bonak, V. A., Umezawa, K. and McColl, S. R. (1990) Blood **76**, 2098–2104
Nasmith, P. E., Mills, G. B. and Grinstein, S. (1989) Biochem. J. **257**, 893–897
O'Shea, J. J., Brown, E. J., Seligmann, B. E., Metcalff, J. A., Frank, M. M. and Gallin, J. I. (1984) J. Immunol. **134**, 2580–2587
Paulson, J. C. (1992) in Adhesion: Its Role in Inflammatory Disease (Harlan, J. M. and Liu, D. Y., eds.), pp. 19–42, W. H. Freeman and Co., New York
Philips, M. R., Buyon, J. P., Winchester, R., Weissmann, G. and Abramson, S. B. (1988) J. Clin. Invest. **82**, 495–501
Reibman, J., Korchak, H. M., Vosshall, L. B., Haines, K. A., Rich, A. M. and Weissmann, G. (1988) J. Biol. Chem. **263**, 6322–6328
Reibman, J., Haines, K. A., Gude, D. and Weissmann, G. (1991a) J. Immunol. **146**, 988–996
Reibman, J., Meixler, S., Lee, T. C., Gold, L. I., Cronstein, B. N., Haines, K. A., Kolasinski, S. L. and Weissmann, G. (1991b) Proc. Natl. Acad. Sci. U.S.A. **88**, 6805–6809
Roos, D., Johan-Muller, J. W. T. and Weening, R. S. (1976) Biochem. Biophys. Res. Commun. **68**, 43–50
Schwartz, B. R., Ochs, H. D., Beatty, P. G. and Harlan, J. M. (1985) Blood **65**, 1553–1556
Serra, M., Bazzoni, F., Bianca, V., Greskowiak, M. and Rossi, F. (1988) J. Immunol. **141**, 2118–2124
Smith, C. W., Rothlein, R., Hughes, B. J., Mariscalco, M. M., Rudloff, H. E., Schmalstieg, F. C. and Anderson, D. C. (1988) J. Clin. Invest. **82**, 1746–1756
Smith, C. W., Kishimoto, T. K., Abbass, O., Hughes, B., Rothlein, R., McIntire, L. V., Butcher, E. and Anderson, D. C. (1991) J. Clin. Invest. **87**, 609–618
Snyderman, R., Smith, C. D. and Verghese, M. W. (1986) J. Leuk. Biol. **40**, 785–800
Stevenson, K. B., Nauseef, W. M. and Clark, R. A. (1987) J. Immunol. **139**, 3759–3763
Todd III, R. F., Arnaout, M. A., Rosin, R. E., Crowley, W. A., Peters, W. A. and Babior, B. M. (1984) J. Clin. Invest. **74**, 1280–1290
Vedder, N. B. and Harlan, J. M. (1988) J. Clin. Invest. **81**, 676–682
Ward, P. A. and Zvaifler, N. J. (1973) J. Immunol. **111**, 1771–1775
Weissmann, G., Smolen, J. E. and Korchak, H. M. (1980) N. Engl. J. Med. **303**, 27–34
Zigmond, S. H. and Hirsch, J. G. (1972) Expt. Cell Res. **73**, 383–393