Thrombospondin receptor expression in human neutrophils coincides with the release of a subpopulation of specific granules

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The extracellular matrix (ECM) protein thrombospondin (TSP) binds specifically to polymorphonuclear leucocyte (PMN) surface receptors and promotes cell adhesion and motility. TSP receptor expression increases 30-fold after activation with the synthetic chemotactic peptide, *N*-formylmethionyl-leucylphenylalanine (FMLP) or the Ca^{2+} ionophore A23187, in combination with cytochalasin B. The expression of TSP receptors was correlated with the exocytosis of both specific and azurophil granules. Newly expressed TSP receptors are not derived from easily mobilized specific granules since agents that trigger some specific granule release [phorbol myristate acetate (PMA), FMLP or ionophore A23187 alone] do not increase TSP receptor expression. In this study we used the anion-channel blocker, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS) to investigate the source of these newly expressed receptors. When PMNs were exposed to cytochalasin B and FMLP or to cytochalasin B and ionophore A23187 in the presence of 30–100 μM -DIDS, TSP receptor expression increased coincidentally with vitamin B_{12} -binding protein release from specific granules. Under these same conditions, the release of the azurophil granule component, myeloperoxidase, was significantly inhibited. Using agonists that cause release of specific granules, or both specific granules and azurophil granules, we determined that DIDS blocked the release of PMA-mobilized specific granules and cytochalasin B plus FMLP- or cytochalasin B plus ionophore A23187-mobilized myeloperoxidase-containing azurophil granules but not specific granules mobilized by cytochalasin B plus FMLP or cytochalasin B plus ionophore A23187. These results suggested that PMNs contain at least two subpopulations of specific granules: one that is easily mobilized, lacks TSP receptors and is inhibitable by DIDS, and one that is difficult to mobilize, contains a large pool of TSP receptors and the release of which is enhanced in the presence of DIDS.

INTRODUCTION

The accumulation of polymorphonuclear leucocytes (PMNs) at sites of inflammation and injury is generally attributed to the presence of specific chemoattractants and increased PMN adherence to endothelial cells and/or subendothelial-extracellular-matrix (ECM) proteins. Chemoattractants not only direct the movement of PMNs but can also trigger superoxide production, increased cell adherence and degranulation [1]. Although increased receptor expression is not a prerequisite for PMN adherence [2,3], it often accompanies activation (degranulation) and may contribute to the formation of new adhesion sites during cell locomotion.

The deposition of the ECM protein, thrombospondin (TSP), along blood vessel walls [4,5] may contribute to the accumulation of PMNs at injury sites. Normally, the levels of TSP in plasma are low (picomolar range), but on platelet activation, TSP released from alpha-granules can lead to an increase in the serum concentration to the nanomolar range [6]. Endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, PMNs and monocytes may also contribute to increased concentrations of TSP [7–13].

In comparison with other ECM proteins such as fibronectin and laminin, TSP is a more transient component of the ECM, appearing early in blood clots and at sites of tissue damage, but disappearing from these sites as the healing process progresses [14,15]. The binding of TSP to endothelial cells [16] or to other ECM proteins in the subendothelial basement membrane [6,17,18] may be crucial for the attachment of PMNs before chemotaxis and diapedesis. The interaction of PMNs with TSP may also trigger degranulation and the release of proteolytic

enzymes necessary for PMN migration through the basement membrane [19]. A significant increase in TSP receptor expression after degranulation could modulate the interaction of PMNs with TSP-coated substrates and TSP-opsonized bacteria to facilitate motility and phagocytosis. Consistent with a role for TSP in PMN functional responses are the observations that TSP binds to specific cell surface receptors [20] and promotes attachment, spreading and motility of human PMNs *in vitro* [21,22].

The intracellular source of many newly expressed PMN cell-surface receptors appears to be specific granules, and in some cases, tertiary granules. CD11b/CD18, CD11c/CD18, T200 and *N*-formylmethionyl-leucylphenylalanine (FMLP) receptor expression correlates with the release of gelatinase (a tertiary and specific granule component), lactoferrin (specific granule) and vitamin B_{12} -binding protein (specific granule) [23–27], thereby suggesting secretory granules as the intracellular source of these receptors. The cosedimentation of these receptors with gelatinase- and vitamin B_{12} -binding protein-containing secretory vesicles in sucrose gradients further supports this hypothesis [24,25,27–29]. Laminin and CR1 receptor expression are also increased under conditions that result in specific granule release [30,31]. However, in contrast with the other receptors studied, CR1 receptors do not appear to co-localize with specific granules [31]. Recently, Singer *et al.* [32] localized receptors for the ECM proteins, laminin, vitronectin and fibronectin, to specific granules. Activation of PMNs with either phorbol myristate acetate (PMA) or FMLP induced fusion of specific granules with the cell membrane and resulted in the increased surface expression of both laminin receptors and CD18 antigens [32].

The ability of cells to regulate receptor expression appears to

Abbreviations used: ECM, extracellular matrix; TSP, thrombospondin; PMN, polymorphonuclear leucocyte; FMLP, *N*-formylmethionyl-leucylphenylalanine; PMA, phorbol myristate acetate; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; PBS, phosphate-buffered saline; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid, RGD, Arg-Gly-Asp.

be central to such processes as oxidant production and chemotaxis [23,33,34]. For example, increasing the number of FMLP receptors on the cell surface can increase the amount of FMLP-mediated oxidant production in PMNs [23,33]. In addition, the movement of PMNs towards gradients of chemotactic factors appears to require reversible adhesive interactions that are sufficiently strong to allow substrate attachment but weak enough to allow subsequent detachment [35]. The removal of occupied receptors from the surface by endocytosis [23,36–38] and appearance of new receptors from preformed intracellular pools provides a mechanism by which PMNs could cycle through rounds of increased and decreased substrate attachment while continuing to move along a chemotactic gradient.

The migration of PMNs into sites rich in ECM proteins may also stimulate PMN phagocytosis of opsonized targets. It has been demonstrated that soluble fibronectin can enhance phagocytosis of complement-opsonized erythrocytes but only if PMNs have been activated first with C5a or FMLP [39]. This indicates that some change in complement receptors (CR1 or CR3) accompanies activation. This change may occur through an increase in the number of receptors expressed from intracellular stores or a redistribution and/or alteration in the conformation of receptors already expressed on the cell surface [31,40–42]. It is possible that both mechanisms, receptor expression and receptor conformation, are required for ECM-mediated phagocytosis.

In previous studies, we have used competitive binding assays to demonstrate that TSP receptor expression was increased 30-fold when cells were activated with cytochalasin B and FMLP [20]. These same conditions result in a significant release of both specific and azurophil granules [43], raising the possibility that TSP receptors could be intracellularly sequestered in azurophil or specific granules, or in both granule types. In the present study, we correlated the release of secretory granule components with the expression of TSP receptors in an attempt to determine the intracellular source of these receptors. The anion-channel blocker 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), which blocked azurophil granule and some specific granule release, provided a useful tool for delineating a subpopulation of specific granules, the release of which correlated with TSP receptor expression.

MATERIALS AND METHODS

Cells

Human PMNs were isolated from human peripheral blood as previously described [44]. Briefly, fresh whole blood was obtained by venipuncture from healthy volunteers and immediately added to acid citrate dextrose. The PMNs were purified by dextran sedimentation followed by hypo-osmotic lysis to remove the majority of erythrocytes and then centrifuged through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) to remove contaminating mononuclear cells. This purification scheme yields at least 98% PMNs with few contaminating platelets and a viability of > 95% as determined by Trypan Blue exclusion.

Materials

Catalase (bovine liver; 11800 units/mg), methionine, sodium azide, FMLP, PMA, DIDS and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and ionophore A23187 was from Calbiochem (San Diego, CA, U.S.A.). ^{57}Co -labelled vitamin B_{12} was purchased from Amersham (Arlington Heights, IL, U.S.A.). Human TSP was isolated from the supernatant of thrombin-activated platelets by affinity chromatography over gelatin-Sepharose and heparin-Sepharose (Pharmacia Fine Chemicals) followed by gel filtration (Bio-Gel A

0.5M; Bio-Rad Laboratories, Richmond, CA, U.S.A.) as outlined in Dixit *et al.* [45]. TSP was concentrated in an ultrafiltration cell (Amicon Corp., Danvers, MA, U.S.A.), and stored at -70°C . For binding studies, TSP was iodinated to a specific radioactivity of $0.1\ \mu\text{Ci}/\mu\text{g}$ using the iodogen method of Fraker and Speck [46].

Assays for secretory granule components

Neutrophils ($5 \times 10^6/\text{ml}$) were suspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ phosphate-buffered saline (PBS) (138 mM-NaCl, 2.7 mM-KCl, 8.1 mM- Na_2HPO_4 , 0.6 mM- CaCl_2 and 1.0 mM- MgCl_2), prewarmed to 37°C for 3 min in the presence or absence of $5\ \mu\text{g}$ of cytochalasin B/ml, and stimulated with the indicated agonist for 10 min at 37°C . Sodium azide (1 mM) was included in vitamin B_{12} -binding protein assays to reduce its destruction by secreted myeloperoxidase [47]. Catalase (5000 units/ml), a scavenger of H_2O_2 , was also included in some vitamin B_{12} -binding protein assays. After agonist treatment, cells were centrifuged at 750 g for 10 min and the supernatants removed. Agonists included 15 ng of PMA/ml, $0.1\ \mu\text{M}$ -FMLP or $0.1\ \mu\text{M}$ -ionophore A23187 without cytochalasin B, and $0.1\ \mu\text{M}$ -FMLP or $10\ \mu\text{M}$ -ionophore A23187 with cytochalasin B. Samples of the supernatants were used for standard determinations of myeloperoxidase [48], an enzyme found exclusively in azurophil granules, and vitamin B_{12} -binding protein [49], a component found exclusively in specific granules. In experiments where DIDS was included, cells were pretreated with $10\text{--}300\ \mu\text{M}$ -DIDS for 3 min, in the presence or absence of cytochalasin B, before stimulation. The values for all data represent the means $\pm$ S.E.M. of three separate experiments done in triplicate.

To determine the reversible nature of DIDS, PMNs ($5 \times 10^6/\text{ml}$) were incubated with DIDS at 37°C for 15 min, centrifuged at 750 g, washed twice in PBS, allowed to recover for 15 min at room temperature, and then pelleted and resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ PBS. These cells were then exposed to FMLP plus cytochalasin B and secretion was evaluated. Untreated PMNs and PMNs exposed to DIDS for 3 min before stimulation served as controls.

In order to test the direct effect of DIDS on the assay system, PMNs were stimulated with $10\ \mu\text{M}$ -ionophore A23187 in the presence of cytochalasin B. The cells were pelleted and the supernatants harvested. Portions of the supernatants were exposed to various concentrations of DIDS for 10 min at 37°C . These supernatants were then compared with supernatants from cells pretreated with DIDS and untreated supernatants.

Binding assays

PMNs ($1 \times 10^7/\text{ml}$) were incubated in blocking buffer [modified Eagle's medium (MEM) containing 1% BSA] for 45 min at 4°C to decrease non-specific TSP binding. Cells were washed once in MEM and resuspended at 2×10^7 cells/ml in binding buffer (MEM containing 0.1% BSA). Binding assays were conducted in $200\ \mu\text{l}$ of binding buffer in $1.5\ \text{ml}$ microfuge tubes that had been precoated with blocking buffer. Specifically, $100\ \mu\text{l}$ of the cell suspension was added to microfuge tubes containing various concentrations of ^{125}I -labelled TSP suspended in $100\ \mu\text{l}$ of binding buffer and incubated for 60 min at 4°C [20]. After incubation, the samples were layered on to a $175\ \mu\text{l}$ sucrose cushion (15%) and centrifuged at 4°C in a Beckman microfuge B (Beckman Instruments, Palo Alto, CA, U.S.A.) at $10000\ g$ for 2 min. The cell pellets were frozen in ethanol/solid CO_2 , cut from the tubes and radioactivity was counted on an LKB 1261 Multigamma gamma counter (LKB, Bromma, Sweden). Specific TSP binding was determined by performing binding assays in the presence of a 100-fold molar excess of unlabelled TSP over a concentration range of $0.5\text{--}16\ \mu\text{g}/\text{ml}$. Catalase (5000 units/ml), a scavenger of

H₂O₂, was included in some binding assays. The number of sites/cell and relative affinity were determined by both Scatchard and non-linear curve-fitting analyses [20].

RESULTS

Unactivated (resting) PMNs have low number of TSP receptors on their cell surface (about 2400±600 receptors/cell), and exposing them to PMA (15 ng/ml), FMLP (0.1 μM) or ionophore

Table 1. Thrombospondin receptor expression on human neutrophils after activation

PMNs were incubated with agonist for 10 min at 37 °C, in the absence or presence of 5 μg of cytochalasin B/ml, washed twice in ice-cold DMEM, and resuspended in MEM containing 1 % BSA for 45 min at 4 °C to decrease non-specific binding. Cells were washed once in MEM and resuspended in MEM+0.1 % BSA containing 0.5–16 μg of ¹²⁵I-labelled TSP/ml, in the absence or presence of a 100-fold excess of unlabelled TSP, and incubated for 60 min at 4 °C. After incubation, the cells were layered on to a sucrose cushion and centrifuged at 10000 g for 2 min at 4 °C. Cell pellets were frozen in ethanol/solid CO₂, cut from the tubes, and radioactivity was counted on a gamma counter. Scatchard analysis of the saturated binding curves was used to estimate the number of TSP binding sites/cell. Cells exposed to 5 μg of cytochalasin B/ml alone for 10 min at 37 °C showed no increase in TSP binding. Values represent means±S.D. for three experiments done in triplicate.

Treatment	TSP binding sites/cell
None	2400 ± 600
15 ng of PMA/ml	2300 ± 450
0.1 μM-FMLP	2350 ± 500
0.1 μM-ionophore A23187	2430 ± 550
Cytochalasin B + 10 μM-ionophore A23187	73000 ± 11000*

* Similar results were observed with cytochalasin B/FMLP activation [20].

Fig. 1. Effect of DIDS on the release of the azurophil granule component, myeloperoxidase

Cells (5 × 10⁶/ml) were preincubated with 5 μg of cytochalasin B/ml in the presence of various concentrations of DIDS in Ca²⁺/Mg²⁺ PBS for 3 min at 37 °C followed by the addition of 0.1 μM-FMLP (●) or 10 μM-ionophore A23187 (○). Incubation was continued for 10 min. Released myeloperoxidase was assayed in the supernatants of centrifuged cell suspensions. PMNs stimulated with cytochalasin B and FMLP or ionophore A23187 served as the 100 % control. Cytochalasin B/FMLP resulted in the release of 50.0±8.5 % and cytochalasin B/A23187 resulted in the release of 56.6±12.0 % of the total cellular myeloperoxidase. Background release was about 1.1±0.3 % for myeloperoxidase. Values represent means±S.E.M. for three separate experiments done in triplicate.

Fig. 2. Effect of DIDS on the release of the specific granule component, vitamin B₁₂-binding protein

PMNs (5 × 10⁶/ml) were preincubated with 5 μg of cytochalasin B/ml in the presence of various concentrations of DIDS in Ca²⁺/Mg²⁺ PBS for 3 min at 37 °C followed by the addition of 0.1 μM-FMLP (●) or 10 μM-ionophore A23187 (○) and incubated for an additional 10 min. The release of vitamin B₁₂-binding protein was assayed in the supernatant of centrifuged cell suspensions. Cytochalasin B/FMLP resulted in the release of 23.0±4.1 % and cytochalasin B/A23187 resulted in the release of 29.7±6.3 % of the total cellular vitamin B₁₂-binding protein. Background release was about 3.6±0.4 % for vitamin B₁₂-binding protein. Values represent means±S.E.M. for three separate experiments done in triplicate.

A23187 (0.1 μM), agents that cause some specific granule release [50], did not alter TSP receptor expression (Table 1) [20]. Interestingly, activation with ‘complete secretagogues’ such as cytochalasin B and FMLP [20] or cytochalasin B and ionophore A23187 (Table 1) increased TSP receptor expression approximately 30-fold. Since azurophil granule release also requires ‘complete secretagogues’ [43], we determined if TSP receptors were derived from azurophil granules.

The anion-channel blocker, DIDS, has been reported to block azurophil granule release from PMNs [51–54] and was therefore evaluated for its utility in analysing degranulation and concomitant TSP receptor expression. In cytochalasin B/FMLP- or cytochalasin B/A23187-treated PMNs, DIDS appeared to block myeloperoxidase release (azurophil granules) in a dose-dependent manner with maximal inhibition at 100–300 μM (Fig. 1). In contrast with these inhibitory effects, DIDS enhanced the apparent release of vitamin B₁₂-binding protein (specific granules) with maximal enhancement at 60 μM (Fig. 2). Another anion-channel blocker, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS), had similar effects on PMN degranulation, although it was somewhat less potent (results not shown). If cells exposed to DIDS were washed twice before the addition of the stimulus, the inhibitory effect was reversed by ≥ 90 % (results not shown).

When PMNs were pretreated with 60 μM-DIDS and stimulated with cytochalasin B/FMLP, conditions that resulted in a 3-fold increase in vitamin B₁₂-binding protein release, TSP receptor expression was enhanced about 3-fold above that measured on activated cells in the absence of DIDS [20]. Specifically, TSP receptor expression increased from 72000±12000 on activated cells to 300000±31000 on cells activated in the presence of DIDS. The coincident increase in TSP receptor expression and vitamin B₁₂-binding protein release in the presence of DIDS and ‘complete’ secretagogues suggested that TSP receptors may not be coming from azurophil but rather from specific granules.

However, as indicated in Table 1, not all agonist-stimulated specific granule release led to increased TSP receptor expression.

Fig. 3. Effect of DIDS on vitamin B₁₂-binding protein release in the absence of cytochalasin B

Cells (5×10^6 /ml) were exposed to 15 ng of PMA/ml ($\blacktriangle$), 0.1 μ M-FMLP ($\bullet$) or 0.1 μ M-A23187 ($\circ$) in Ca^{2+} /Mg $^{2+}$ PBS in the presence of various concentrations of DIDS for 10 min at 37 °C. After incubation, cells were centrifuged and the supernatants assayed for vitamin B₁₂-binding protein. PMNs stimulated with PMA, FMLP or ionophore A23187 served as the 100 % control. PMA resulted in the release of $44.4 \pm 16.0\%$, FMLP resulted in the release of $16.6 \pm 4.3\%$ of the total cellular vitamin B₁₂-binding protein. Background release was about $3.4 \pm 0.3\%$ for vitamin B₁₂-binding protein. Under these same conditions, myeloperoxidase release was $0.3 \pm 0.2\%$ of the total cellular myeloperoxidase. Values represent means $\pm$ S.E.M. for three separate experiments done in triplicate.

Fig. 4. Direct effect of DIDS on vitamin B₁₂-binding protein

Cells (5×10^6 /ml) were exposed to 5 μ g of cytochalasin B/ml/10 μ M-ionophore A23187 (a) or 5 μ g of cytochalasin B/ml/0.1 μ M-FMLP (b) in the absence ($\circ$, $\triangle$) or presence ($\bullet$, $\blacktriangle$) of DIDS, and the supernatants were recovered. The supernatants obtained in the absence of DIDS were then exposed to various concentrations of DIDS for 10 min at 37 °C. The supernatants from DIDS-treated cells ($\bullet$, $\blacktriangle$) were then compared with DIDS-treated supernatants ($\circ$, $\triangle$) to determine the effect of DIDS on the assay for vitamin B₁₂-binding protein. PMNs stimulated with cytochalasin B and ionophore A23187 served as the 100% control. Cytochalasin B/FMLP resulted in the release of $25.0 \pm 6.2\%$ and cytochalasin B/A23187 resulted in the release of $30.5 \pm 6.1\%$ of the total cellular vitamin B₁₂-binding protein. Background release was about $2.8 \pm 0.6\%$ for vitamin B₁₂-binding protein. Values represent means $\pm$ S.E.M. for three separate experiments done in triplicate.

In an attempt to distinguish between populations of specific granules, we examined the effect of DIDS on granule release using PMA, ionophore A23187 or FMLP in the absence of cytochalasin B, conditions that promote the release of only easily mobilized specific granules. In the absence of cytochalasin B, DIDS had variable effects on agonist-stimulated vitamin B₁₂-binding protein release (Fig. 3). Specifically, DIDS inhibited PMA-mediated vitamin B₁₂-binding protein release but slightly enhanced FMLP-mediated release. The ionophore A23187-stimulated release of vitamin B₁₂-binding protein was relatively unaffected by DIDS treatment.

Since previous reports indicated that relatively high concentrations ($\geq 100 \mu\text{M}$) of anion-channel blockers such as DIDS and SITS interfered with the measurement of parathyroid hormone from parathyroid cells and β -glucuronidase from neutrophils [52,55], we wanted to evaluate whether DIDS directly affected the assays for myeloperoxidase and vitamin B₁₂-binding protein. Thus we activated PMNs with the 'complete secretagogues', cytochalasin B/A23187 or cytochalasin B/FMLP, and exposed the supernatants to increasing concentrations of DIDS before conducting the assay for each granule component. The assay for vitamin B₁₂-binding protein was not inhibited by DIDS since increasing concentrations of DIDS did not interfere with the measurement of vitamin B₁₂-binding protein in the supernatants of PMNs activated with either cytochalasin B plus 10 μ M-ionophore A23187 or cytochalasin B plus 0.1 μ M-FMLP (Figs. 4a and 4b respectively). Therefore our results using vitamin B₁₂-binding protein as a specific granule marker demonstrated that DIDS blocked PMA-mobilized specific granule release and appeared to enhance FMLP-mediated specific granule release in both the absence and presence of cytochalasin B.

Although it had been previously reported that DIDS blocked the exocytosis of azurophil granules [53], the direct effect of DIDS on the measurement of myeloperoxidase was not demonstrated. In contrast with the assay for β -glucuronidase (results

not shown), increasing concentrations of DIDS did not affect measurements of myeloperoxidase in the supernatants of PMNs activated with either cytochalasin B plus 10 μ M-ionophore A23187 or cytochalasin B plus 0.1 μ M-FMLP (Figs. 5a and 5b respectively). However, treatment of PMNs with $\geq 100 \mu\text{M}$ -DIDS in the presence of cytochalasin B followed by ionophore A23187 or FMLP totally blocked myeloperoxidase release. These experiments demonstrated that DIDS did not interfere with the detection of myeloperoxidase in cell supernatants but did block azurophil granule release. Since 50–70 % inhibition of azurophil granule release was observed at 60 μM -DIDS, a concentration that correlated with enhanced TSP receptor expression, these data suggested that TSP receptors were not derived from azurophil granules. Thus there appear to be at least two subpopulations of specific granules; one that is mobilized by PMA, lacks TSP receptors and is inhibited by DIDS, and one that requires complete secretagogues for mobilization, contains TSP receptors and whose release appears to be enhanced in the presence of DIDS.

Interestingly, the correlation between inhibition of azurophil granule release and the enhanced release of vitamin B₁₂-binding protein in the presence of DIDS suggested that azurophil granule components could be degrading a fraction of the secreted vitamin B₁₂-binding protein in the absence of DIDS. Since sodium azide,

Fig. 5. Direct effect of DIDS on myeloperoxidase activity

Cells (5×10^6 /ml) were exposed to 5 μ g of cytochalasin B/ml/10 μ M-A23187 (a) or 5 μ g of cytochalasin B/ml/0.1 μ M-FMLP (b) in the presence (●, ▲) or absence (○, △) of DIDS and the supernatants were collected. Supernatants obtained in the absence of DIDS were exposed to various concentrations of DIDS for 10 min at 37 °C. The supernatants from DIDS-treated cells (▲, ●) were compared with DIDS-treated supernatants (△, ○) to determine the effect of DIDS on both azurophil granule release and the assay system. PMNs stimulated with cytochalasin B/A23187 or FMLP served as 100 % control. Cytochalasin B/A23187 resulted in the release of $59.3 \pm 9.0\%$ and cytochalasin B/FMLP resulted in the release of $53.8 \pm 10.0\%$ of the total cellular myeloperoxidase. Background release was about $1.2 \pm 0.4\%$ for myeloperoxidase. Values represent means $\pm$ S.E.M. for three separate experiments done in triplicate.

an inhibitor of myeloperoxidase, did not increase detectable vitamin B₁₂-binding protein to the same extent as DIDS (a 20–40 % increase compared with a 100–200 % increase; results not shown), we used 5000 units of catalase/ml to scavenge H₂O₂ generated during azurophil granule release. When catalase was present during cytochalasin B and FMLP- or cytochalasin B and ionophore A23187-induced degranulation, the values for vitamin B₁₂-binding protein obtained were similar to those obtained in the presence of 60 μ M-DIDS (Table 2). Catalase alone does not affect granule release (results not shown). These results indicated that the enhanced vitamin B₁₂-binding protein release observed in the presence of DIDS correlated with inhibition of azurophil granule release and accompanying oxidant production.

The apparent increase in TSP receptor expression in the presence of DIDS also correlated with inhibition of azurophil granule release, suggesting that TSP receptors might also be sensitive to oxidant damage. When catalase was present during cytochalasin B and FMLP-induced degranulation, an additional 3-fold increase in receptor expression was observed (above the 30-fold increase seen with cytochalasin B/FMLP alone) indicating that TSP receptors were susceptible to oxidant damage (Table 3). Substituting azide for catalase did not result in the same observed increase in receptor expression (a 1.3-fold increase for azide compared with a 2.7-fold increase for catalase) suggesting that this damage is mediated primarily through H₂O₂

Table 2. Effect of the H₂O₂ scavenger, catalase, on release of vitamin B₁₂-binding protein

Results are expressed as percentage of control with 100 % representing the amount of vitamin B₁₂-binding protein released in the presence of 5 μ g of cytochalasin B/ml and FMLP or ionophore A23187. PMNs were simultaneously incubated in 60 μ M-DIDS or 5000 units of catalase/ml. Values represent means $\pm$ S.D. for three experiments done in triplicate.

Treatment	Percentage of control release
Cytochalasin B+0.1 M-FMLP	100
Cytochalasin B+0.1 M-FMLP + 60 μ M-DIDS	304 $\pm$ 108
Cytochalasin B+0.1 μ M-FMLP + catalase	248 $\pm$ 80
Cytochalasin B+10 μ M-ionophore A23187	100
Cytochalasin B+10 μ M-ionophore A23187 + 60 μ M-DIDS	267 $\pm$ 94
Cytochalasin B+10 μ M-ionophore A23187 + catalase	202 $\pm$ 47

Table 3. Effect of catalase on cytochalasin B/FMLP-stimulated thrombospondin receptor expression

PMNs were activated with 5 μ g of cytochalasin B/ml+0.1 M-FMLP for 10 min at 37 °C in the presence of DIDS or catalase. Binding assays to determine TSP binding sites/cell were conducted as outlined in Table 1. Control cells were PMNs activated with cytochalasin B/FMLP in the absence of DIDS or catalase. Values represent means $\pm$ S.D. for three experiments done in triplicate.

Treatment	TSP binding sites/cell
Control	74000 $\pm$ 13000
60 μ M-DIDS	300000 $\pm$ 31000
5000 units of catalase/ml	200000 $\pm$ 26000

rather than myeloperoxidase/H₂O₂ (results not shown). The apparent enhancement of both vitamin B₁₂-binding protein release and TSP receptor expression was not the result of DIDS inhibition of PMN superoxide generation since 60 μ M-DIDS did not affect superoxide production in response to the agonists used in this study (results not shown). This is consistent with reports from several other laboratories showing that 100 μ M-DIDS had no effect on the respiratory burst elicited by a number of different agonists [51–53,56].

DISCUSSION

Evidence indicating that PMN cell surface receptors are regulated by diverse molecular mechanisms has been mounting over the past several years. For example, secretagogues such as PMA, FMLP or C5a increase FMLP and casein receptor expression but decrease the expression of C5a receptors [57]. PMN activation also results in the loss of gp100^{me1-14}, a PMN adhesion receptor related to lymphocyte MEL-14, from the cell surface with a concomitant increase in CD11b/CD18 receptors [58,59]. These examples demonstrate overt changes in receptor expression, i.e. receptors are either lost from or added to the cell surface. However, other examples of receptor modulation demonstrate more subtle variations. Exposing PMNs to cytochalasin B alone at 37 °C triggers about a 2-fold increase in FMLP receptors whereas FMLP plus cytochalasin B increases receptor expression 3-fold [57]. In contrast with FMLP receptors, cyto-

chalasin B alone has no effect on casein receptors but cytochalasin B plus FMLP increases casein receptor expression about 15-fold. PMA causes approximately a 2-fold increase in FMLP receptors and about a 4-fold increase in casein receptors. Since one mechanism for regulating receptor expression on PMNs involves the translocation of receptors from intracellular storage sites to the plasma membrane during cell activation, one interpretation of these data is that different agonists mobilize different subpopulations of secretory granules.

The possibility that the same receptor might reside within two different granule populations in PMNs did not evolve until fairly recently. Initial observations suggested that increased CD11b/CD18 and CD45 receptor expression occur under conditions that do not correlate with the release of specific granule contents. These experiments suggest that at least one additional intracellular pool exists for these receptors [24–26]. Recently, it was demonstrated that gelatinase-containing tertiary granules release their contents under conditions that lead to increased FMLP, CD11b/CD18 and CD45 receptor expression [24,60]. This evidence further suggested that tertiary granules might serve as a second intracellular source of these receptors. Although gelatinase-containing tertiary granules and vitamin B₁₂-binding protein-containing specific granules differentially sediment on sucrose gradients during PMN subcellular fractionation [24,61], the existence of distinct 'tertiary' granules has recently been questioned. Double-label immunolocalization experiments using antibodies against lactoferrin, a specific granule marker, and gelatinase demonstrated co-localization of these two antigens in 80 % of the specific granules [62]. Since tertiary granules were originally defined using cell fractionation and functional criteria, it is conceivable that tertiary granules constitute a subpopulation of specific granules. Although there appears to be a graded degranulation response of PMNs to various stimuli, the possible heterogeneity of specific granules has not been fully explored. In one of the few reports addressing specific granule heterogeneity, Perez *et al.* [63] took advantage of the differential calcium sensitivity of lactoferrin and vitamin B₁₂-binding protein release to demonstrate the heterogeneous functional nature of PMN specific granules.

In addition to specific and tertiary granules, another potential intracellular source of CD11b/CD18 and FMLP receptors has recently been identified in PMNs [64]. This intracellular compartment has been characterized by its latent alkaline phosphatase activity. The concentration of FMLP (nanomolar range) required to release 90 % of this latent alkaline phosphatase activity results in only a minimal release of vitamin B₁₂-binding protein and gelatinase. Ultrastructural localization of alkaline phosphatase activity in resting PMNs has identified a granule type distinct from previously described azurophil and specific granules [65]. Whether this granule type also contains some specific granule components remains to be determined.

In the present study, we used DIDS to distinguish between two subpopulations of specific granules in PMNs, one that was easily mobilized, lacked TSP receptors and was sensitive to DIDS, and a second that was difficult to mobilize, contained a large pool of TSP receptors and the release of which was unaffected by DIDS. In earlier studies on PMNs, it was reported that anion-channel blockers such as DIDS and SITS interfered with immune complex- or calcium ionophore-mediated lysosomal enzyme release [51]. The interpretation of these findings was that anion-channel blockers somehow interfered with lysosomal membrane-plasma membrane fusion. A subsequent publication showed that DIDS and SITS inhibited anion fluxes in PMNs which correlated with inhibition of degranulation [52]. Unfortunately, these studies relied exclusively on assays for lysozyme and β -glucuronidase to assess granule release, assays that are impeded by the presence of

DIDS. DIDS has also been shown to inhibit myeloperoxidase release significantly in response to cytochalasin B and FMLP, leukotriene B₄, platelet-activating factor or PMA [53]. However, the fate of specific granules was not addressed. Other groups have reported that DIDS blocks CD11b/CD18 and CR1 receptor expression, presumably by blocking the release of specific granules [3,54]. These studies on both degranulation and receptor expression indicate that anion-channel blockers generally inhibit degranulation. However, by assaying for both specific granule and azurophil granule contents, as well as measuring TSP receptor expression, we have identified a specific granule subpopulation that is insensitive to DIDS.

Recently, some of the older studies that reported using anion-channel blockers to block exocytosis have been reassessed. A recent study examining thrombin-induced secretion in platelets demonstrated that DIDS and SITS inactivated the agonist rather than blocking exocytosis as had been previously reported [55]. This same study also indicated that anion-channel blockers interfered with the detection of lysozyme and β -glucuronidase, but not degranulation, in PMNs. Unfortunately, these authors only evaluated the effect of a single concentration of DIDS on the measurement of β -glucuronidase and compared this finding with published data from other laboratories rather than comparing it with PMNs exposed to DIDS in their own assay system. We also noted that DIDS affected the assay for β -glucuronidase, but the inhibition was not as great as that seen when PMNs were pretreated with DIDS and then activated (results not shown). This suggested that DIDS might also be blocking exocytosis. Along these same lines, Korchak *et al.* [52] noted that, although concentrations of SITS and DIDS greater than 100 μ M inhibited the assays for β -glucuronidase and lysozyme, they were able to correct for this inhibitory effect making it possible to demonstrate that these anion-channel blockers did inhibit degranulation.

In contrast with the effect of DIDS on the assay systems for lysozyme and β -glucuronidase, no effect was observed in our measurements of vitamin B₁₂-binding protein or myeloperoxidase. The inhibition of myeloperoxidase release at $\geq 100 \mu$ M-DIDS indicated that cytochalasin B/A23187-stimulated azurophil granule release was being blocked under these conditions. Whether this population of azurophil granules is the same as those containing β -glucuronidase is unresolved at this time. Interestingly, DIDS affected specific granule release (in the absence of cytochalasin B) differentially depending on the specific agonist employed. DIDS inhibited PMA-mediated vitamin B₁₂-binding protein release and appeared to increase FMLP-mediated release, but had no effect on ionophore A23187-mediated release. These findings demonstrated that DIDS does not globally inhibit PMN granule release. The differential effect of DIDS on specific granule release indicated that different regulatory mechanisms may be involved in the release of subpopulations of specific granules. In general, our findings support earlier reports that anion-channel blockers inhibit PMN degranulation, but also suggest the existence of granule populations the exocytosis of which is not susceptible to DIDS treatment.

The apparent enhancement in vitamin B₁₂-binding protein release and TSP receptor expression in the presence of DIDS correlates with the inhibition of azurophil granule release. Damaging oxidants, specifically H₂O₂, released during degranulation may destroy agonists, secretory products and receptors alike. Gresham *et al.* [66] demonstrated that Arg-Gly-Asp (RGD)-mediated phagocytosis in PMNs was enhanced 2–3-fold in the presence of catalase, a scavenger for H₂O₂. The inclusion of catalase also resulted in a 9-fold increase in RGD binding to PMNs, suggesting that the effect of catalase was on ligand-receptor interactions rather than on phagocytosis [66]. Similar results were observed by Winn *et al.* [67] when they

examined serum-activated zymosan stimulated oxidant production. PMNs treated with micromolar concentrations of H_2O_2 had a suppressed response to serum-activated zymosan whereas exogenously added catalase removed the inhibitory effect of H_2O_2 . When catalase alone was added to PMNs before stimulation with serum-activated zymosan, their response was enhanced significantly above control cells. The authors' interpretation of this finding was that H_2O_2 produced extracellularly during the respiratory burst normally exerts a negative feedback effect, decreasing the overall magnitude of the response.

H_2O_2 generated during phagocytosis has also been shown to oxidize FMLP, a process inhibited by catalase [68]. Inactivation of a fraction of FMLP during degranulation may provide some explanation for the elevated concentrations of vitamin B_{12} -binding protein observed in the presence of DIDS, but a more likely explanation is a direct effect of H_2O_2 on vitamin B_{12} -binding protein itself. Clark and Borregaard [47] demonstrated that about 50 % of the vitamin B_{12} -binding protein released from PMNs during activation is destroyed by damaging oxidants. The inclusion of catalase with cells during activation increased the recovery of vitamin B_{12} -binding protein to about 80 %. The inability of catalase to scavenge released H_2O_2 efficiently [69] would explain the incomplete recovery of vitamin B_{12} -binding protein in the above study [47] and the lower values we obtained for both vitamin B_{12} -binding protein release and TSP receptor expression in the presence of catalase as compared with DIDS (compare values within Tables 2 and 3). Since DIDS does not appear to affect oxidant production directly, as measured by O_2^- generation, our data would indicate that some azurophil-granule-derived component or components contribute to oxidant damage. The inability of azide to duplicate the catalase effect suggests that this component is not solely myeloperoxidase. An alternative interpretation of our data would involve a direct effect of DIDS on H_2O_2 to neutralize oxidative damage. At present, this issue remains unresolved.

The functional data obtained in our studies and those of other investigators indicate that there is a certain amount of heterogeneity among specific granules. Cell fractionation studies have demonstrated size and density differences among specific granules [70] but overlapping granule constituents have hindered the identification of distinct specific granule subpopulations. The co-localization of specific granule constituents and sequestered membrane proteins (i.e. laminin and TSP receptors) should provide a powerful tool for characterizing specific granule subpopulations. The differential mobilization of azurophil and specific granules, and the ability to alter this mobilization with DIDS, can be used to isolate and characterize subpopulations of specific granules.

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REFERENCES

- Sandborg, R. R. & Smolen, J. E. (1989) in *Pathology Reviews: Biology of Disease* (Rubin, E. & Damjanov, I., eds.), pp. 133–153, Humana Press, Clifton, NJ
- Schleiffenbaum, B., Moser, R., Patarroyo, M. & Fehr, J. (1989) *J. Immunol.* **142**, 3537–3545
- Vedder, N. B. & Harlan, J. M. (1988) *J. Clin. Invest.* **81**, 676–692
- Wight, T. N., Raugi, G. J., Mumby, S. M. & Bornstein, P. (1985) *J. Histochem. Cytochem.* **33**, 295–302
- Raugi, G. J., Mullen, J. S., Bark, D. H., Okada, T. & Mayberg, M. R. (1990) *Am. J. Pathol.* **137**, 179–185
- Lawler, J. (1986) *Blood* **67**, 1197–1209
- Mosher, D. F., Doyle, M. J. & Jaffe, E. A. (1982) *J. Cell Biol.* **93**, 343–348
- Raugi, G. J., Mumby, S. M., Abbott-Brown, P. & Bornstein, P. (1982) *J. Cell Biol.* **95**, 351–354
- Jaffe, E. A., Ruggiero, J. T., Leung, L. L. K., Doyle, M. J., McKeown-Longo, P. J. & Mosher, D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 998–1002
- Sage, H., Farin, F. M., Striker, G. E. & Fisher, A. B. (1983) *Biochemistry* **22**, 2148–2155
- Wikner, N. E., Dixit, V. M., Frazier, W. A. & Clark, R. A. F. (1987) *J. Invest. Dermatol.* **88**, 207–211
- Jaffe, E. A., Ruggiero, J. T. & Falcone, D. J. (1985) *Blood* **65**, 79–84
- Kreis, C., La Fleur, M., Menard, C., Paquin, R. & Beaulien, A. D. (1989) *J. Immunol.* **143**, 1961–1968
- Murphy-Ullrich, J. E. & Mosher, D. F. (1985) *Blood* **66**, 1098–1104
- Raugi, G. J., Olerud, J. E. & Gown, A. M. (1987) *J. Invest. Dermatol.* **89**, 551–554
- Murphy-Ullrich, J. E. & Mosher, D. F. (1987) *J. Cell Biol.* **105**, 1603–1611
- Mumby, S. M., Raugi, G. J., & Bornstein, P. (1984) *J. Cell Biol.* **98**, 646–652
- Galvin, N. J., Vance, P. M., Dixit, V. M., Fink, B. & Frazier, W. A. (1987) *J. Cell Biol.* **104**, 1413–1422
- Wachtfogel, Y. T., Abrams, W., Kucich, U., Weinbaum, G., Schapira, M. & Colman, R. W. (1988) *J. Clin. Invest.* **81**, 1310–1316
- Suchard, S. J., Boxer, L. A. & Dixit, V. M. (1991) *J. Immunol.* **147**, 651–659
- Suchard, S. J., Burton, M. J., Dixit, V. M. & Boxer, L. A. (1991) *J. Immunol.* **146**, 3945–3952
- Mansfield, P. J., Boxer, L. A. & Suchard, S. J. (1990) *J. Cell Biol.* **111**, 3077–3086
- Fletcher, M. P., Seligmann, B. E. & Gallin, J. I. (1982) *J. Immunol.* **128**, 941–948
- Lacal, P., Pulido, R., Sanchez-Madrid, F. & Mollinedo, F. (1988) *J. Biol. Chem.* **263**, 9946–9951
- Lacal, P., Pulido, R., Sanchez-Madrid, F., Cabanas, C. & Mollinedo, F. (1988) *Biochem. Biophys. Res. Commun.* **154**, 641–647
- Petrequin, P. R., Todd, R. F., III, Duvall, L. J., Boxer, L. A. & Curnutte, J. T., III. (1987) *Blood* **69**, 605–610
- Jones, D. H., Schmalstieg, F. C., Hawkins, H. K., Burr, B. L., Rudloff, H. E., Krater, S., Smith, C. W. & Anderson, D. C. (1989) in *Leukocyte Adhesion Molecules: Structure, Function, and Regulation* (Springer, T. A., Anderson, D. C., Rosenthal, A. S. & Rothlein, R., eds.), pp. 106–124, Springer-Verlag, New York
- Jesaitis, A. J., Naemura, J. R., Painter, R. G., Sklar, L. A. & Cochrane, C. G. (1982) *Biochim. Biophys. Acta* **719**, 556–568
- Fletcher, M. P. & Gallin, J. I. (1983) *Blood* **62**, 792–799
- Yoon, P. S., Boxer, L. A., Mayo, L. A., Yang, A. Y. & Wicha, M. S. (1987) *J. Immunol.* **138**, 259–265
- O'Shea, J. J., Brown, E. J., Seligmann, B. E., Metcalf, J. A., Frank, M. M. & Gallin, J. I. (1985) *J. Immunol.* **134**, 2580–2587
- Singer, I. I., Scott, S., Kawka, D. W. & Kazazis, D. M. (1989) *J. Cell Biol.* **109**, 3169–3182
- Pike, M. C., Wicha, M. S., Yoon, P., Mayo, L. & Boxer, L. A. (1989) *J. Immunol.* **142**, 2004–2011
- Springer, T. A. & Anderson, D. C. (1986) *Ciba Found. Symp.* **118**, 102–126
- Wilkinson, P. C. (1982) in *Chemotaxis and Inflammation*, 2nd edn., Churchill Livingstone, London
- Janeczek, A. H., Marasco, W. A., Van Alten, P. J. & Walter, R. J. (1989) *J. Cell Sci.* **94**, 155–168
- Jesaitis, A. J., Naemura, J. R., Painter, R. G., Sklar, L. A. & Cochrane, C. G. (1983) *J. Biol. Chem.* **258**, 1968–1977
- Daukas, G. & Zigmond, S. H. (1985) *J. Cell Biol.* **101**, 1673–1679
- Pommier, C. G., O'Shea, J., Chused, T., Yancey, K., Frank, M. M., Takahashi, T. & Brown, E. J. (1984) *J. Exp. Med.* **159**, 137–151
- Berger, M., O'Shea, J., Cross, A. S., Folks, T. M., Chused, T. M., Brown, E. J. & Frank, M. M. (1984) *J. Clin. Invest.* **74**, 1566–1571
- Detmers, P. A., Wright, S. D., Olsen, E., Kimball, B. & Cohn, Z. A. (1987) *J. Cell Biol.* **105**, 1137–1145
- Mercurio, A. M. & Shaw, L. M. (1988) *J. Cell Biol.* **107**, 1873–1880
- Smolen, J. E. (1987) in *In Vitro Methods for Studying Secretion: Secretion from Neutrophils: A Critique of Modern In Vitro Techniques* (Poisner, A. M. & Trifaro, J. M., eds.), pp. 29–43, Elsevier, Amsterdam
- Curnutte, J. T., Babior, B. M. & Karnovsky, M. L. (1979) *J. Clin. Invest.* **63**, 637–647

45. Dixit, V. M., Grant, G. A., Santoro, S. A. & Frazier, W. A. (1984) *J. Biol. Chem.* **259**, 10100–10105
46. Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
47. Clark, R. A. & Borregaard, N. (1985) *Blood* **65**, 375–381
48. Bretz, U. & Baggiolini, M. (1974) *J. Cell Biol.* **63**, 251–269
49. Dewald, B. & Baggiolini, M. (1986) *Methods Enzymol.* **132**, 267–277
50. Bentwood, B. J. & Henson, P. M. (1980) *J. Immunol.* **124**, 855–862
51. Korchak, H. M., Eisenstat, B. A., Hoffstein, S. T., Dunham, P. B. & Weissmann, G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2721–2725
52. Korchak, H. M., Eisenstat, B. A., Smolen, J. E., Rutherford, L. E., Dunham, P. B. & Weissmann, G. (1982) *J. Biol. Chem.* **257**, 6916–6922
53. Smith, R. J., Bowman, B. J. & Iden, S. S. (1984) *Biochem. Biophys. Res. Commun.* **120**, 964–972
54. Tauber, A. I. & Goetzl, E. J. (1981) *J. Immunol.* **126**, 1786–1789
55. Vostal, J. G., Reid, D. M., Jones, C. E. & Shulman, N. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5839–5843
56. Mollinedo, F., Gajate, C. & Schneider, D. (1991) *Mol. Cell. Biochem.* **105**, 49–60
57. Bender, J. G., Van Epps, D. E. & Chenoweth, D. E. (1987) *J. Immunol.* **139**, 3028–3033
58. Jutila, M. A., Rott, L., Berg, E. L. & Butcher, E. C. (1989) *J. Immunol.* **143**, 3318–3324
59. Kishimoto, T. K., Jutila, M. A., Berg, E. L. & Butcher, E. C. (1989) *Science* **245**, 1238–1241
60. Petrequin, P. R., Todd, R. F., III, Smolen, J. E. & Boxer, L. A. (1986) *Blood* **67**, 1119–1125
61. Dewald, B., Bretz, R. & Baggiolini, M. (1982) *J. Clin. Invest.* **70**, 518–525
62. Hibbs, M. S. & Bainton, D. F. (1989) *J. Clin. Invest.* **84**, 1395–1402
63. Perez, D. H., Marder, S., Elfman, F. & Ives, H. E. (1987) *Biochem. Biophys. Res. Commun.* **145**, 976–981
64. Borregaard, N., Miller, L. J. & Springer, T. A. (1987) *Science* **237**, 1204–1206
65. Kobayashi, T. & Robinson, J. M. (1991) *J. Cell Biol.* **113**, 743–756
66. Gresham, H. D., Goodwin, J. L., Allen, P. M., Anderson, D. C. & Brown, E. J. (1989) *J. Cell Biol.* **108**, 1935–1943
67. Winn, J. S., Guille, J., Gebicki, J. M. & Day, R. O. (1991) *Biochem. Pharmacol.* **41**, 31–36
68. Tsan, M. F. & Denison, R. C. (1981) *J. Immunol.* **126**, 1387–1389
69. Ohno, Y. & Gallin, J. I. (1985) *J. Biol. Chem.* **260**, 8438–8446
70. Rice, W. G., Kinkade, J. M. & Parmley, R. T. (1986) *Blood* **68**, 541–555

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