Albumin Inhibits Neutrophil Spreading and Hydrogen Peroxide Release by Blocking the Shedding of CD43 (Sialophorin, Leukosialin)

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Abstract. Spreading of neutrophils on protein-coated surfaces is a pivotal event in their ability to respond to soluble, physiologic agonists by releasing large amounts of hydrolases and oxidants. Using neutrophils plated on serum-, fibrinogen- or fibronectin-coated surfaces, we investigated the effect of human serum albumin (HSA) on spreading-dependent neutrophil responses. HSA suppressed the respiratory burst of neutrophils in response to tumor necrosis factor-α (TNF), complement component C5a or formylated peptide, but not phorbol myristate acetate. HSA was suppressive only if added before the onset of the respiratory burst, and suppression was reversed when HSA was removed. Likewise, HSA selectively and reversibly inhibited TNF-induced cell spreading and the associated fall in cAMP. However, HSA did not hinder TNF-induced cell adherence to the same protein-coated surfaces.

We investigated cell surface sialoproteins as modulators of cell spreading and as targets for the anti-spreading action of HSA. Oxidation of the cell surface with periodate followed by reduction with ^{3}H-borohydride and immunoblotting with specific mAbs helped identify the predominant sialoprotein on human neutrophils as CD43 (sialophorin, leukosialin). Treatment of neutrophils with *C. perfringens* sialidase desialylated CD43, markedly enhanced the ability of the cells to respond to TNF by spreading and undergoing a respiratory burst, and antagonized the ability of HSA to inhibit these responses. TNF-treated, adherent neutrophils shed CD43, and this was blocked by HSA, but not by ovalbumin. Exogenous neutrophil elastase removed CD43 from the neutrophil surface. HSA blocked the actions of both sialidase and elastase on CD43. In contrast, ovalbumin did not block the action of sialidase on CD43, and HSA did not inhibit the ability of sialidase to hydrolyze a synthetic substrate. These results suggested that HSA might bind CD43. In fact, the extracellular portion of CD43 bound to HSA-Sepharose, but not to ovalbumin- or glycylglycine-Sepharose. Finally, two mAbs recognizing different epitopes on CD43 mimicked HSA's inhibitory effects on neutrophil function.

Thus, HSA can dissociate attachment of neutrophils from spreading. This dissociation may help neutrophils migrate along a chemotactic gradient, while decreasing their release of oxidants. CD43, a long, rigid molecule with a markedly negative charge, antagonizes neutrophil spreading. HSA appears to inhibit spreading-dependent neutrophil functions by binding to CD43 and interfering with the ability of neutrophils to shed it.

THE anti-infectious and pro-inflammatory functions of neutrophils are usually discharged after the cells have stopped circulating and have adhered to each other or to platelets, endothelium, parenchyma, stromal cells, or proteins of extracellular matrix. This has motivated a number of investigators to explore the biology of neutrophils adherent to model biological surfaces. A simple model consists of neutrophils plated on plastic or glass coated with plasma, serum, fibrinogen, fibronectin, vitronectin, laminin, or thrombospondin (12, 18, 23, 30, 31, 33, 39–42, 54, 63, 65), or bearing a confluent monolayer of endothelial cells (39) or myocytes (15). A major finding to emerge from such studies is that soluble, physiologic poly- or oligopeptides such as tumor necrosis factor (TNF)[1]-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1 (MIP-1), interleukin-8 (IL-8), C5a, and formylated methionylleucylphenylalanine (fMLF) act as potent, cytochalasin-independent secretagogues under these conditions, whereas the same agents act as weak, cytochalasin-dependent agonists when tested with neutrophils in suspension.

1. *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; fMLF, formylated methionylleucylphenylalanine; HSA, human serum albumin; KRPG, Krebs-Ringer phosphate buffer with glucose; TNF, tumor necrosis factor.

The secretory response of adherent, cytokine-stimulated neutrophils depends on their expression of $\beta 2$ integrins (CD11/CD18) (23, 41, 63). It is not understood how neutrophils integrate signals from adhesion receptors and agonist receptors before committing to secretion. The following events all appear to be essential: a sustained fall in cAMP (41); spreading of the cells (41); reorganization of the actin-based cytoskeleton to form focal adhesions (41); and tyrosine phosphorylation of proteins associated with these adhesions (18). We have speculated (18, 41) that the reorganization of the actin-based cytoskeleton from a subcortical meshwork into focal adhesions may remove a barrier to exocytosis and provide a nidus for the colocalization of integrins, functionally important tyrosine phosphoproteins, and components of the respiratory burst oxidase (43, 66).

It has not been clear how the behavior of neutrophils in such culture systems relates to more complex physiologic conditions. Our efforts to explore this question revealed that plasma and serum suppress cell spreading-dependent neutrophil responses to physiologic agonists. A major component of serum contributing to suppression proved to be albumin. Human serum albumin (HSA) acted at an early step distinct from the binding of the agonists to their receptors. These observations directed our attention to molecular events by which one such agonist, TNF-α, induces spreading of neutrophils, and mechanisms by which HSA may interfere with this process.

The ability of neutrophils to spread appears to be restricted by their expression of sialoproteins at the cell surface, perhaps because of electrostatic repulsion associated with the glycans' negative charge. Treatment with exogenous sialidase reduces the negative charge of neutrophils (10) and increases their adhesiveness (10, 24). Treatment of neutrophils with secretagogues is accompanied by a reduction in their negative charge (20). Neutrophils activated in this way express a sialidase on their surface (10). A sialidase inhibitor blocks the decrease in the negative charge of the cells and their increased adhesion to plastic (10). Activated neutrophils shed the sialoprotein CD43 (sialophorin; leukosialin) (7, 32, 55), a process antagonized by protease inhibitors (7, 55). Exogenous sialidase markedly potentiates the ability of an exogenous protease to cleave CD43 from lymphocytes (51). Thus, for neutrophils to spread, they may have to mobilize sialidase and proteases for a concerted attack on surface sialoproteins.

In the present study, we observed that treatment with sialidase markedly enhanced the ability of TNF to induce cell spreading and a respiratory burst in neutrophils plated on serum- or fibronectin-coated plastic, and antagonized the ability of HSA to inhibit these responses. The predominant sialoprotein on the surface of neutrophils was CD43. Treatment with TNF led to shedding of CD43, but only in neutrophils adherent to a protein-coated surface. HSA but not ovalbumin blocked the TNF-induced loss of CD43. Exogenous sialidase and elastase each caused the loss of CD43-associated epitopes from the cell surface, and HSA blocked their ability to do so. In contrast, ovalbumin did not protect CD43 from sialidase, and HSA did not protect a synthetic substrate. Thus the target of HSA appeared to be CD43 itself. This interpretation was supported by the specific binding of extracellular CD43 to HSA, and by the ability of two anti-CD43 mAbs to mimic HSA's inhibitory effects on neutrophil function.

These results suggest that albumin may act physiologically to damp massive secretory responses by neutrophils in the circulation and in the stages of inflammation characterized by formation of exudates. CD43 hinders spreading by neutrophils. HSA appears to inhibit spreading-dependent neutrophil functions by binding to CD43 and interfering with the ability of neutrophils to shed it.

Materials and Methods

Cell Preparation and Functional Responses

Neutrophils were isolated from normal donors' heparinized venous blood on a one-step, modified Ficoll–Hypaque gradient and resuspended in ice-cold Krebs-Ringer phosphate buffer with glucose (KRPG) as described (41). For measurements of H_2O_2 release and adherence, lysis of the few contaminating erythrocytes was avoided, since it risks activating the cells. For experiments analyzing cell-surface sialoproteins, erythrocytes were lysed by resuspending the cell pellets in 0.2% NaCl, followed 30 s later by addition of an equal volume of 1.6% NaCl; the procedure was repeated if necessary. Polystyrene Primaria® tissue culture plates (6-mm-diam wells) (Falcon Labware; Becton-Dickinson & Co., Oxnard, CA) were precoated with FBS, fibrinogen or fibronectin and washed as described (41). Plates containing reaction mixture consisting of 100 μl/well KRPG with 0.44 purpurogallin U/ml HRP, 4 nmol scopoletin, 1 mM NaN_3 (to inhibit myeloperoxidase), and the indicated test agents were prewarmed to 37°C in air. Test agents were PMA (100 ng/ml), recombinant human TNF (Genentech, Inc., South San Francisco, CA) at the indicated concentrations, *N*-formylated methionylleucylphenylalanine (fMLF) (10^{-6} M), recombinant human complement component C5a (Pfizer, Inc., Groton, CT) (10^{-8} M), or buffer alone. Respiratory burst assays were begun by adding 1.5×10^4 cells/well. The HRP-catalyzed oxidation of scopoletin by H_2O_2 was recorded at 15-min intervals for 2 h in a plate-reading fluorometer (41). To test reversibility of HSA's effects, plates were flicked empty after the 60-min reading, flooded with warm KRPG, flicked again, then repleted with 100 μl warm reaction mixture per well. Cell spreading was monitored at 30-min intervals by inspection on an inverted phase-contrast microscope.

For assays of adherence, the cell number was increased to 10^5/well. At 30 and 60 min, separate plates were flicked empty and flooded with 0.9% saline, three times. The residual adherent cell protein was measured as described (41).

Cell spreading was recorded photographically using glutaraldehyde-fixed monolayers derived from 2×10^5 cells plates on protein-coated glass coverslips as detailed (41).

Cyclic AMP was measured by radioimmunoassay in lysates of 7–9 $\times$ 10^5 neutrophils per 16-mm-diam FBS-coated wells as reported (41).

HSA Preparations

Electrophoretically pure HSA was obtained from Armour Pharmaceuticals (Kankakee, IL), Calbiochem Corp. (San Diego, CA), and The New York Blood Center and protein concentration determined by the method of Lowry et al. (35) using BSA as a standard.

To remove lipids, 30 mg HSA in 100 μl was extracted with 50 μl each of chloroform and methanol. After centrifugation at 8,000 *g* for 4 min, the organic layer was reserved and the precipitate re-extracted in the same manner. The pooled organic layers were evaporated under N_2 and the residue resuspended by vortexing in 100 μl KRPG. A control organic phase was prepared by evaporating 100 μl each of chloroform and methanol and vortexing the residue with 100 μl KRPG. The twice-extracted precipitate from the aqueous layer was likewise redissolved/resuspended in 100 μl KRPG. All three preparations were then compared in a dose-dependent manner for their ability to suppress the release of H_2O_2 induced by TNF but not by PMA.

To prepare HSA-derived peptides, 2 mmol HSA was dissolved in 5 ml of 200 mM Tris, 6 M guanidine HCl, 20 mM EDTA, pH 8.5, and made 0.3–3.0 mM in 2-mercaptoethanol. A fivefold molar excess of 2-vinylpyridine (Aldrich Chemical Co., Milwaukee, WI) was added and the solution incubated for 30 min at 37°C, followed by extensive dialysis against 0.9% saline in the cold using tubing with a 1-kD cut-off (Spectropor Industries, Los Angeles, CA). The dialysate was brought to 0.1 N HCl and a 50–500-fold molar excess of CNBr added. After 40 h at room temperature in the dark, the preparation was dialyzed extensively against 20 mM acetic acid, 100 mM NaCl, pH 2.9. Alternatively, reduction and alkylation were

omitted, and 6.6 mmol HSA was dissolved in 87 ml 70% formic acid. A 50-fold molar excess of CNBr was added. After 20 h at room temperature, the solution was diluted with 1 vol of H_2O, chilled, and brought to 70–75% saturation with $(NH_4)_2SO_4$. The pellet was collected by centrifugation, resuspended in PBS, and dialyzed against 0.9% saline. The supernate of the dialysate was concentrated in polyethylene glycol 8,000 and brought to 6 M in urea. Reversed phase chromatography was carried out on 6-mg samples at room temperature on a Waters system (Waters Assoc., Milford, MA) with a Vydac phenyl column (25 cm × 4.6 mm internal diam, 5 mm pore size) (Separation Group, Hesperia, CA), eluting at 1 ml/min with 0.1% trifluoroacetic acid in water and a stepped gradient ranging from 0–100% acetonitrile. After the protein concentration of each fraction was measured by the Bradford assay (Bio Rad Labs, Hercules, CA), equal amounts of protein were subjected to SDS-PAGE analysis and the fractions were dried in a Speedvac (Savant Instruments, Inc., Farmingdale, NY). For assay, the residues were redissolved in 5 mM glycine, 2.3 mM HCl, pH 3.0. Fractions with similar Coomassie blue staining patterns were pooled, diluted in the same glycine-HCl buffer and added to the H_2O_2 assay in a volume of 10 μl/well. Controls included a protein-free fraction eluting late from the phenyl column and processed in the same way, and glycine-HCl buffer alone.

Modification and Detection of Cell-Surface Sialoproteins

Where indicated, neutrophils suspended in KRPG on ice were exposed to 50 mU/ml of *C. perfringens* sialidase (Type X; Sigma Immunochemicals, St. Louis, MO) for 30 min and then plated. These conditions were chosen after preliminary experiments tested the effect of varying the concentration of sialidase (0.5, 5.0, or 50 mU/ml), temperature (4° or 37°C), and duration of treatment (10, 20, 30, or 60 min before plating the cells, with or without wash-out). A wide range of conditions gave similar results.

To radiolabel cell-surface sialoproteins (19), 2×10^7 neutrophils/ml per sample in KRPG were treated on ice with sialidase or buffer alone, then made 1 mM in sodium periodate (Baker Chemical Co., Phillipsburg, NJ). 5 min later, 0.2 ml of 0.1 M glycerol in KRPG was added and the cells were washed three times in KRPG by centrifugation in the cold. The cells were then rotated for 30 min at room temperature with 0.5 mCi/tube of sodium ^{3}H-borohydride (sp. act. 468.7 mCi/mmol) (Dupont, Wilmington, DE) and washed three times. The pellets were lysed in 1% Triton X-100 with 5.7 mM diisopropylfluorophosphate (DFP), 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM PMSF in 10 mM Tris, pH 7.4 (lysis buffer). Protein was measured by the Bradford method in the supernatant after centrifugation at 8,000 *g* for 10 min. Equal amounts of protein per lane were subjected to 10% SDS-PAGE, followed by autoradiography and immunoblot. Radioactivity did not interfere with chemiluminescent detection of immunoblots, for which the film was exposed for only 1–5 min (see below).

For testing the effects of exposure to endogenous *C. perfringens* sialidase or purified neutrophil elastase (Calbiochem Corp.) on CD43, 10^7 neutrophils in 1 ml KRPG per condition were suspended in FBS-precoated polypropylene microfuge tubes. For sialidase treatment, neutrophils were kept on ice; for treatment with elastase, they were kept at 37°C. Enzyme-free controls showed some spontaneous shedding of CD43 at the higher temperature (not shown). After 20 min, the cells were washed three times in KRPG. The pellets were lysed in 125 μl lysis buffer and processed for immunoblot as described above.

Adherent cells were prepared by plating ~10^7 neutrophils in 7 ml of H_2O_2 reaction mixture in one 100-mm petri dish per condition tested. The dishes had been precoated with 3 ml each of FBS. After addition of test agents, the cells were incubated at 37°C in air and inspected periodically on an inverted phase-contrast microscope until the TNF-treated cells began to spread, typically at 30–60 min. At this point, all plates were placed on ice and the nonadherent cells were collected. The adherent cell monolayer was rinsed twice with KRPG to remove HSA and the rinse was pooled with the nonadherent cells. The nonadherent cells were washed twice by centrifugation and resuspension in KRPG to remove HSA. After a final centrifugation, the pellets were each resuspended in the same 125-μl aliquot of lysis buffer that had been incubated for 15 min on ice with the corresponding monolayer of adherent cells. Suspended cells were prepared with the same amounts of cells and medium as for adherent cells, but were tumbled in FBS-precoated tubes at 37°C until being washed and lysed as above.

For immunoblot, samples were transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The filters were blocked with 5% nonfat milk (Carnation Corp., Los Angeles, CA), 0.05% Tween-20 in PBS, and then exposed to 1:2,000 dilution of mouse IgG1 anti-CD43 mAb MEM59 (43) (SolarCare Technologies, Bethlehem, PA), or a 1:1,000 dilution of mouse IgG1 anti-CD43 mAb L10 (51), a kind gift of E. Remold-O'Donnell (Center for Blood Research, Boston, MA) in 2.5% milk, 0.025% Tween-20 in PBS for 2 h at room temperature. After three washes with 0.05% Tween-20 in PBS, the antibody-stained bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL).

Fluorogenic Assays for Sialidase and Elastase

C. perfringens sialidase (0.3 mU/well) was incubated with 4-methylumbelliferyl-*N*-acetyl-neuraminic acid (Sigma Immunochemicals) (10, 49) at 0.5 mM in 100 ml of 0.1 M sodium acetate buffer, pH 4.6, in microtest plate wells, along with various concentrations of HSA. After 15 min at 37°C, samples were alkalinized with 50 μl 1 N NaOH, product solubilized with 50 μl ethanol, and fluorescence recorded in a fluorometric plate reader (Flow Laboratories, Inc., McLean, VA). As described (6), purified neutrophil elastase (0.1 μg) was incubated at 37°C with Ala-Ala-Pro-Val-7-amido-α-methyl coumarin (0.25 mM) (Sigma Immunochemicals) in 100 μl of 150 mM NaCl with 20 mM Tris, pH 8.0 in the presence of various concentrations of HSA and fluorescence recorded at 5-min intervals from 5–30 min. Both reactions were evaluated in comparison to a standard curve prepared with 4-methyl-umbelliferone (Sigma Immunochemicals). A separate standard curve was constructed for each concentration of HSA.

Affinity Purification of CD43 on HSA-Sepharose

Cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was coupled at a ratio of 333 mg gel to 7.5 mg HSA, ovalbumin or glycylglycine and blocked according to the manufacturer's instructions. Soluble, extracellular domains of CD43 were collected by treating 2×10^8 neutrophils in 1 ml KRPG with 5 mM DFP for 10 min on ice and then bringing the cells to 42 ml in H_2O_2 reaction mixture containing 2 mM leupeptin, 1 μg/ml soybean trypsin inhibitor, 0.5 μg/ml aprotinin, and 5 mM iodoacetamide, distributing them in six tubes and tumbling them with 100 ng/ml PMA for 30 min at 37°C. After centrifugation, one third of the pooled supernatants was passed over each of the three columns at room temperature. The effluent of each column was recycled on the same column twice. Total dwell time on the columns was 2–3 h. The effluent of the third pass was reserved and pooled with the first 2 ml eluting during a wash with 20 ml of KRPG. These pools were concentrated in Centricon tubes with a 10 kD cut-off (Amicon Division of W. R. Grace, Danvers, MA). Material bound to the washed columns was then eluted with 1 M NaCl in KRPG in 20 fractions of 0.5 ml each. Those fractions containing protein were concentrated by filtration as above. All concentrated samples at equivalent volumes on a per-cell basis were then subjected to SDS-PAGE and immunoblot with anti-CD43 mAbs.

Other Procedures and Reagents

Peptides were sequenced at The Rockefeller University Protein Sequencing Facility (New York) by Edman degradation. The limulus amebocyte lysate assay kit for bacterial lipopolysaccharide was from Whittaker Biologicals, Walkersville, MD. Mouse IgG1 anti-human neutrophil Fc receptor mAb 3G8 (16) was a kind gift of J. Unkeless (Mt. Sinai Medical Center, New York). Mouse ascites containing IgG1 anti-gelsolin mAb and other reagents whose sources have not been specified above or in ref. 41 were from Sigma Immunochemicals.

Results

HSA Selectively and Reversibly Suppresses the Respiratory Burst of Adherent Neutrophils

Neutrophils were plated on FBS-coated plastic in KRPG and observed over a 2-h period. No H_2O_2 was released unless the cells were exposed to PMA, TNF, or fMLF. All three agonists provoked a massive respiratory burst, although the response to the two physiologic agonists began after a long delay (Figure 1 *A*), consistent with previous observations (39, 41, 42). When the medium was supplemented with 1.5 mg/ml HSA, the response to PMA was augmented: instead of approaching a plateau by 75 min, it remained nearly linear through 120 min. This excluded that HSA was toxic to the

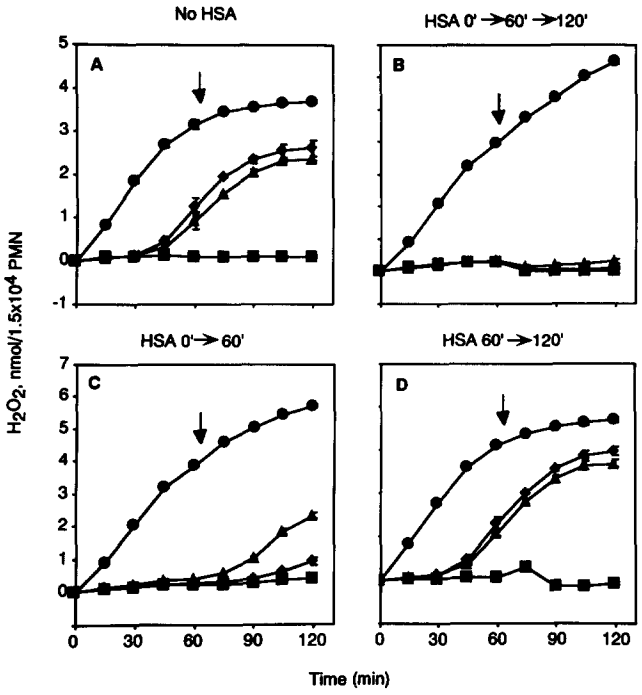

Figure 1. HSA suppresses the neutrophil respiratory burst selectively and reversibly, but only if added before its onset. Neutrophils were exposed to PMA (100 ng/ml) (●), TNF (10 ng/ml) (▲), fMLF (10^{-6} M) (♦) or buffer alone (■), and were additionally incubated either with buffer alone or HSA (1,500 μg/ml) for the first 60 min of the experiment. At that point (*vertical arrows*), all plates were flicked empty, rinsed, repleted with fresh reaction mixture and exposed to HSA or not for the remaining 60 min, as indicated above each panel. PMA, TNF, and fMLF were not added back after the plates were washed. Data are means ± SEM of triplicates. In this and subsequent figures, some of the error bars fall within the symbols denoting the means.

neutrophils or interfered with the assay system. In contrast, HSA abolished the release of H_2O_2 in response to TNF or fMLF (Fig. 1 *B*). The suppressive effect of HSA was reversible, because when the medium was changed at 60 min and replaced with HSA-free medium, H_2O_2 release in response to TNF began after a typical lag (Fig. 1 *C*). TNF was only added to the medium in the first stage of the assay. When the HSA was removed, TNF was not replaced, so that the cells were presumably responding to TNF that had bound earlier in the presence of HSA. This suggested that HSA did not interfere with the binding of TNF to its cell surface receptors. Finally, HSA only blocked the TNF- and fMLF-induced respiratory burst if added before its onset. Thus, HSA had no effect if it was added 60 min after TNF or fMLF (Fig. 1 *D*). This indicated that the critical action of HSA was confined to an early event in the response to neutrophils to TNF and fMLF. Results were similar when TNF and fMLF were replaced by an agonistic mAb against the 55-kD TNF receptor (HT-9, 10 μg/ml) (5) or by 10^{-8} M recombinant C5a (not shown). That HSA inhibited H_2O_2 release in response to four different agonists was further evidence that HSA did not act by interfering with their binding.

Suppression of the TNF-induced respiratory burst was dependent on the concentration of HSA added to the medium. With TNF at 1 ng/ml, neutrophils plated on FBS-coated plastic were half-maximally suppressed by 46 μg/ml HSA

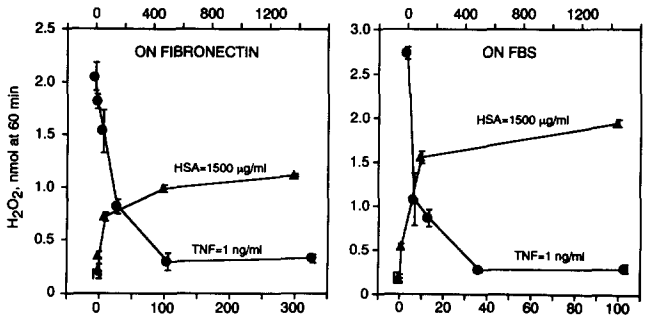

Figure 2. Concentration dependence of HSA-mediated suppression of the TNF-induced respiratory burst, and partial reversal of suppression by increasing concentrations of TNF. Neutrophils were plated in wells precoated with fibronectin (*left panel*) or FBS (*right panel*). At a fixed concentration of TNF (1 ng/ml) (▲), HSA suppressed H_2O_2 release in a dose-dependent manner, as indicated by the scale above each panel (μg/ml). At a fixed concentration of HSA (1,500 μg/ml) (●), suppression was partly overcome by increasing concentrations of TNF, as indicated by the scale below each panel (ng/ml). Data are means ± SEM of triplicates.

(Fig. 2). The IC_{50} ranged up to 180 μg/ml HSA in other experiments. Suppression by HSA did not depend on the use of FBS to coat the plastic, but was also evident with fibronectin (IC_{50}, 105 μg/ml HSA) (Fig. 2) or fibronectin (not shown) as coatings. At a concentration of HSA threefold greater than that required to produce maximal suppression of the response to 1 ng/ml TNF, increasing the TNF concentration to 100 ng/ml restored H_2O_2 release to 53% (on fibronectin) or 72% (on FBS) of the level seen without HSA (Fig. 2).

Table I summarizes results of 22 experiments. H_2O_2 release in response to TNF was always suppressed, but never abolished, even when HSA was added at 5 mg/ml. At 15 mg/ml, HSA interfered with the assay used to detect H_2O_2 (not shown) (56). In contrast, ovalbumin (Table I), lactoferrin and transferrin (not shown) neither suppressed the response to TNF nor augmented the response to PMA. Thus the actions of HSA in this system were not simply a consequence of adding protein.

HSA Reversibly Suppresses TNF-induced Spreading

Because HSA suppressed a functional response of neutrophils at an early step, and because spreading is an early event essential for the functional response tested, we examined the effect of HSA on TNF-induced spreading of neutrophils. The design of these experiments was patterned on that in Fig. 1. Within 30 min of exposure to TNF, neutrophils were well spread in an irregular and often crescentic shape (Fig. 3 *a*). Spreading proceeded to a disk-like shape by 60 min, even though the medium was changed at 30 min and TNF was not replaced (Fig. 3 *c*). The continuous presence of HSA from time 0 blocked spreading at both 30 min (Fig. 3 *b*) and 60 min (Fig. 3 *d*), although the neutrophils remained adherent. In contrast, HSA had no effect on spreading if its addition was delayed until after spreading had already commenced (HSA added from 30–60 min; Fig. 3 *e*). Conversely, cells prevented from spreading for 30 min in the presence of HSA began to spread at the normal rate when HSA was removed, even though fluid-phase TNF had been removed along with the HSA and was not added back (Fig. 3 *f*).

Table I. HSA but Not Ovalbumin Suppresses Hydrogen Peroxide Release Induced by TNF but Not by PMA

Agent	Concentration	Measure	TNA	TNF	PMA
	μg/ml		*1 ng/ml*	*10 ng/ml*	*100 ng/ml*
HSA	0	nmol*	2.20 ± 0.18(15)	2.52 ± 0.16(13)	2.96 ± 0.16(22)
	10–15	% of control‡	98 ± 6(8)	ND	106 ± 0(3)
	30–50	"	79 ± 9(9)	85 ± 4(3)	102 ± 4(5)
	100–150	"	38 ± 8(11)	46 ± 11(5)	94 ± 2(5)
	300–500	"	12 ± 2(12)	27 ± 8(7)	102 ± 4(9)
	1,000–1,500	"	15 ± 7(5)	22 ± 5(9)	126 ± 3(18)
	3,000–5,000	"	15 ± 4(2)	15 ± 3(3)	133 ± 6(7)
Ovalbumin	1,500	"	103 ± 5(3)	ND	92 ± 3(4)

* Nmol H_2O_2 released/1.5 × 10^4 neutrophils 60–120 min after plating the cells in wells precoated with FBS, fibronectin or fibrinogen. The time-point selected for each experiment was that at which the TNF response reached ~90% of maximum (mode, 75 min). Means ± SEM, where each datum is the mean of an experiment performed in triplicate, for the number of experiments in parentheses. Spontaneous release (without TNF or PMA) averaged 0.14 ± 0.03 (n = 23 experiments) and was unaffected by HSA or ovalbumin.
‡ Results for the HSA- or OVA-treated sets expressed as a percent of the corresponding HSA-, OVA-free set in the same experiment. Means ± SEM for the number of experiments in parentheses.

Results with PMA as agonist were strikingly different than with TNF. Neutrophils treated with PMA proceeded directly to disk-like spreading, as illustrated in references 18 and 41. HSA hastened rather than inhibited PMA-induced spreading (not shown).

HSA Does Not Affect TNF-induced Cell Attachment

Morphologic observations like those recorded in Fig. 3 suggested that the number of neutrophils adherent to FBS-, fibrinogen-, or fibronectin-coated plastic or glass was unaffected by HSA, even though the cells were prevented from spreading. This was quantified by subjecting monolayers of neutrophils to vigorous washing and measuring the residual adherent cell protein. By this test, adherence of neutrophils increased more than 10-fold in response to TNF, whether or not HSA was present (Table II). This result provided a third piece of evidence that HSA did not act by interfering with the binding of TNF to its receptors.

HSA Blocks the TNF-induced Fall in cAMP

Spreading of TNF-treated neutrophils depends on a fall of cAMP that persists at least until spreading starts (41). HSA not only blocked the ability of TNF to induce cell spreading, but also prevented the associated fall in cAMP, measured 45 min after plating the cells (Table III).

Suppressive Activity Is Intrinsic to HSA and Some of its Proteolytic Fragments

Since HSA can act as a carrier for other molecules (37), it was important to determine whether the suppressive activity on spreading-related responses of neutrophils was intrinsic to HSA. The experiments summarized in Table I were carried out with HSA from three different suppliers. All lots were free of bacterial lipopolysaccharide by a limulus amebocyte lysate assay sensitive to 10–20 pg/ml. All three preparations were pure by the criterion that only a single band was visible by Coomassie blue staining after running 10 μg HSA on SDS-PAGE (not shown). As monitored by suppression of H_2O_2 release in response to TNF, chloroform-methanol extraction of HSA distributed all bioactivity to the protein phase and none to the lipid phase. Nonspecific toxicity was excluded, because H_2O_2 release in response to PMA was unaffected (not shown).

Finally, HSA was digested with CNBr with or without prior reduction and alkylation, and chromatographed by a variety of methods. We only isolated one CNBr fragment of HSA in pure form. This species, which migrated at 20.0 kD on SDS-PAGE, was identified by NH_2-terminal sequencing (XTAFHDNEET) as arising from the CNBr cleavage site at Met^{124} and beginning with the adjacent Cys^{125}; it presumably continued to the next Met, which follows amino acid 298 (20.0 kD) (14). Peptide 124–298, which is the largest CNBr fragment of HSA, accounting for 30% of its mass, was inactive in the H_2O_2 bioassay (not shown). However, reversed phase HPLC on a phenyl column (Fig. 4 *A*) did yield a pool of fractions highly active at suppressing H_2O_2 release induced by TNF (Fig. 4 *B*) and not by PMA (Fig. 4 *C*). This pool was enriched in polypeptides migrating at 47, 37, 32, 22, 12, and 10 kD, along with smaller species migrating at the dye front (not shown) (the three larger species are incomplete digestion products). The active polypeptide pool was eightfold more potent on a weight basis than HSA itself (Fig. 4 *B*), perhaps reflecting the increased molar concentration of the active peptide(s) relative to the parent molecule. Although these results fell short of identifying bioactive region(s) of HSA, they suggested that the suppressive activity of HSA was intrinsic to certain portions of the molecule and not others.

Sialidase Enhances Cell Spreading and the Respiratory Burst Induced by TNF and Prevents Inhibition by HSA

We turned next to the mechanism of TNF-induced spreading of neutrophils, in order to see how HSA might interfere with it. We began by testing the possibility that spreading is regulated by cell-surface sialoproteins.

When neutrophils were plated on FBS-coated dishes after pretreatment with *C. perfringens* sialidase, they remained nonadherent, spherical, and refractile by phase-contrast microscopy (not shown) and released no H_2O_2 over a 120-min period of observation (Fig. 5 *A*). Moreover, sialidase had no effect on the rapid cell spreading and H_2O_2 release induced by PMA (Fig. 5 *A*). In contrast, pretreatment with sialidase markedly enhanced responses to TNF. In the experiment il-

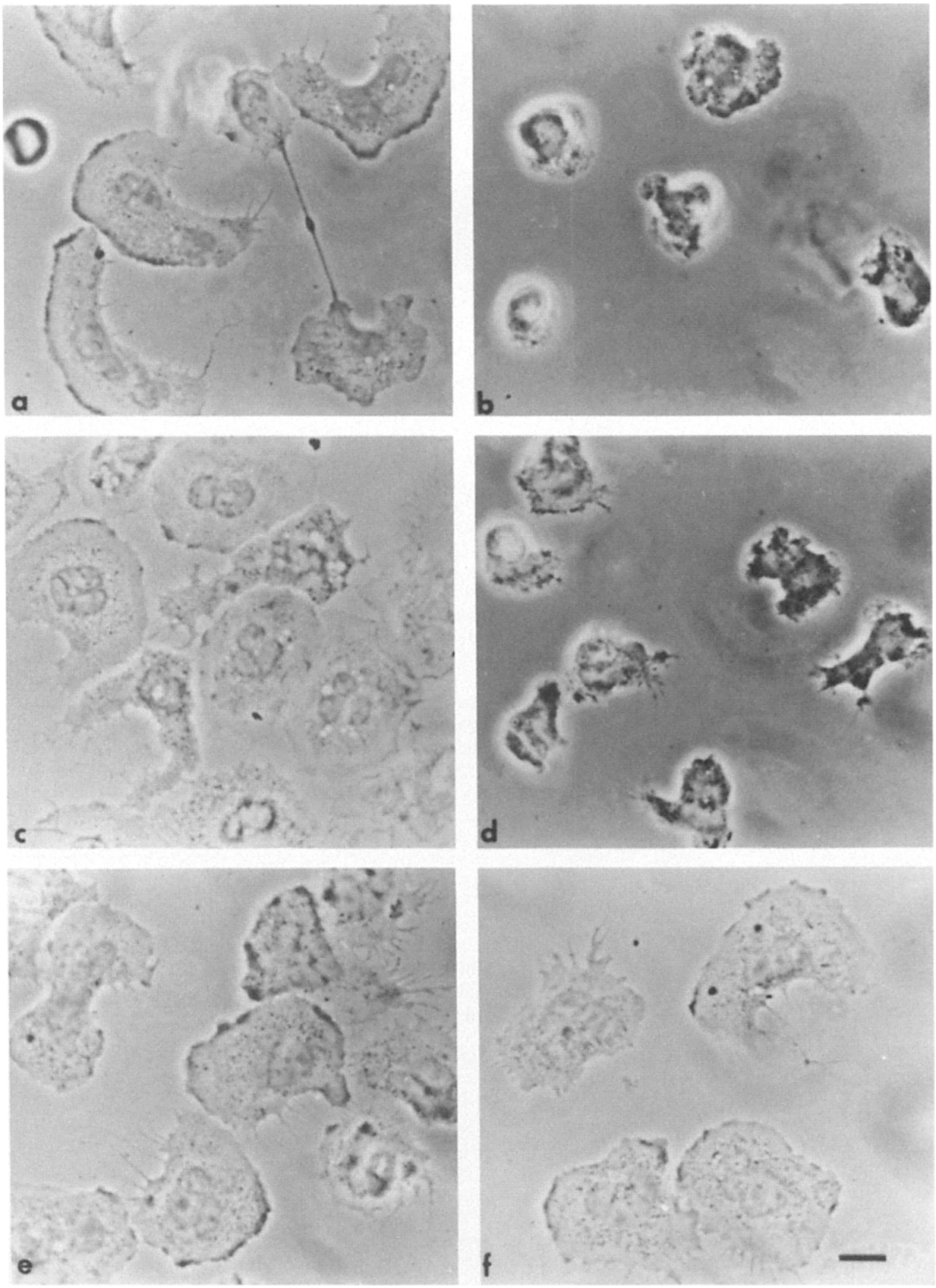

Figure 3. HSA reversibly inhibits TNF-induced cell spreading, but only if added before its onset. TNF (10 ng/ml) was added at time 0 to neutrophils on FBS-coated glass coverslips. At 30 min, coverslips were either fixed or transferred to fresh medium without TNF. (*a*) No HSA added; cells fixed at 30 min. The cell spreading was TNF-induced, since without TNF, the neutrophils remained phase-bright and spherical, as illustrated in Fig. 6 *I* of ref. 18. (*b*) HSA (1,500 μg/ml) added at time 0; cells fixed at 30 min. (*c*) No HSA; cells fixed at 60 min. (*d*) HSA added at time 0 and again at 30 min; cells fixed at 60 min. (*e*) HSA first added at 30 min; cells fixed at 60 min. (*f*) HSA added at time 0, but not again at 30 min; cells fixed at 60 min.

*Table II. Lack of Effect of HSA on Adherence of Neutrophils to FBS-coated Plates**

HSA	Stimulus		
	None	TNF‡	PMA§
μg/ml	*μg adherent cell protein/well*		
0	0.9 ± 0.2(4)‖	10.4 ± 0.6(4)	10.6 ± 0.8(4)
500	0.3 ± 0.2(3)	9.9 ± 0.3(3)	10.8 ± 0.3(3)
1,500	0.8 ± 0.0(2)	8.3 ± 0.2(2)	10.2 ± 0.2(2)

* Adherence was measured 60 min after plating 10^5 neutrophils per FBS-coated well.
‡ 10 ng/ml.
§ 100 ng/ml.
‖ Means ± SEM for mean results from (*n*) experiments, each in triplicate.

*Table III. HSA Blocks the TNF-induced Fall in cAMP in Adherent Neutrophils**

HSA	Stimulus	
	None	TNF‡
μg/ml	*Percent of control*	
0	100(3)§	66.4 ± 2.6(3)
1,500	91.7 ± 3.9(4)	96.4 ± 10.6(4)

* cAMP was measured 45 min after plating neutrophils in FBS-coated wells.
‡ 10 or 100 ng/ml.
§ Means ± SEM for the mean results from (*n*) experiments, each in triplicate. Values for the controls (no HSA, no TNF) averaged 0.41 ± 0.06 pmol/10^6 neutrophils.

lustrated in Fig. 5 *B*, spreading (not shown) and H_2O_2 release to an effective concentration of TNF (1 ng/ml) began 23 min earlier than without sialidase. Moreover, with sialidase, TNF was able to induce cell spreading and a respiratory burst at 0.1 ng/ml, a concentration ineffective on neutrophils not exposed to sialidase (Fig. 5 *B*). Thus, sialidase and TNF acted synergistically.

HSA-mediated suppression of the respiratory burst in response to TNF was largely forestalled in neutrophils pretreated with sialidase (Fig. 5 *C*). Likewise, sialidase restored TNF-induced cell spreading in the presence of HSA (not shown).

These findings suggested that cell-surface sialoproteins might normally counteract TNF-induced spreading and the respiratory burst, and might also be a target for the inhibitory actions of HSA on these two processes.

CD43 Is the Predominant Cell Surface Sialoprotein of Neutrophils

CD43 is a myeloid-specific mucin that represents the predominant cell surface sialoprotein on lymphocytes (50). CD43 is expressed by neutrophils (7, 8, 17, 52, 55), but the proportion of neutrophil cell surface sialoproteins represented by CD43 has apparently not been clearly established. Cell surface sialic acids can be specifically labeled by briefly exposing cells to low concentrations of sodium periodate at 0°C and reducing the resulting aldehydes with tritiated borohydride (19). Application of this procedure to neutrophils

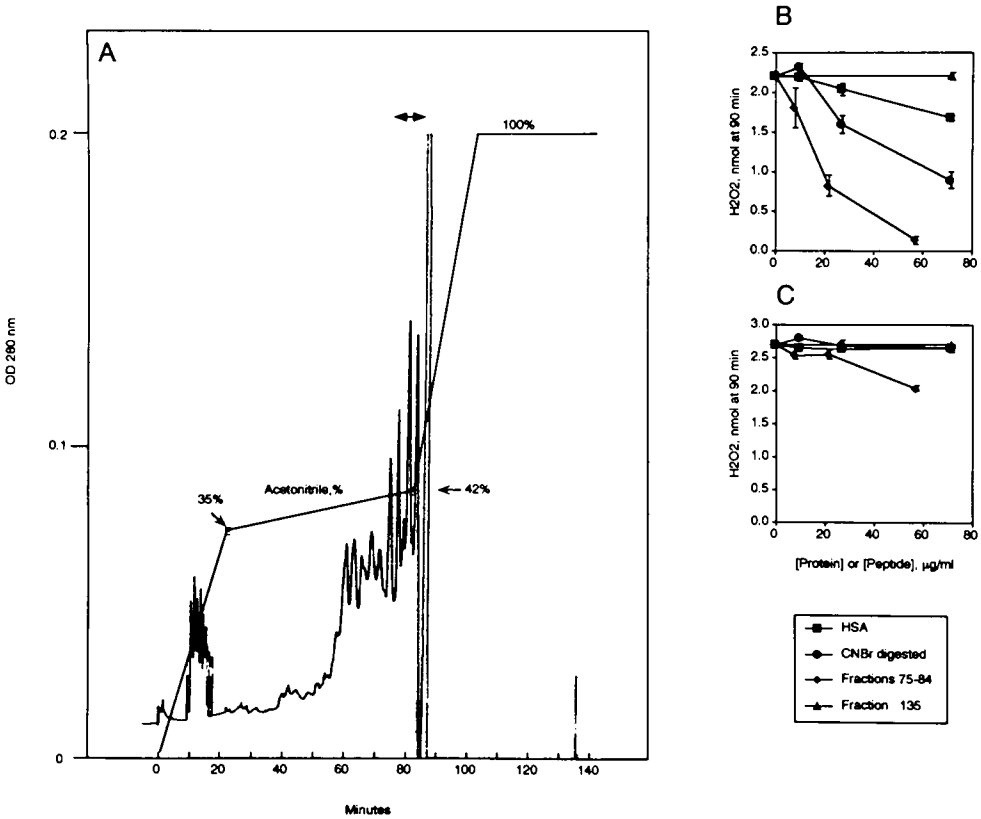

Figure 4. A subset of HSA-derived CNBr fragments is enriched in suppressive activity for the TNF-induced respiratory burst. (*A*) Reversed phase HPLC chromatogram following injection of 6 mg of HSA-derived CNBr fragments. The phenyl column was eluted at 1 ml/min with 0.1% trifluoroacetic acid in water and the indicated gradient of acetonitrile. All protein-containing fractions were subjected to SDS-PAGE and those with similar Coomassie blue staining patterns were pooled. Maximal bioactivity was recovered in the pool of fractions 75–84 (eluting with 42–58% acetonitrile; double-headed arrow). (*B*) Potency of pool 75–84 in suppression of neutrophil H_2O_2 release induced by TNF (1 ng/ml). As a control for nonspecific effects, fraction 135, which lacked measurable protein, was processed and diluted in the same way as the highest concentration of pool 75–84. In addition, glycine-HCl buffer alone had no effect on neutrophil morphology or respiratory burst (not shown). Means ± SEM for triplicates. (*C*) Lack of effect of pool 75–84 on neutrophil H_2O_2 release induced by PMA (100 ng/ml). Means ± SEM for triplicates.

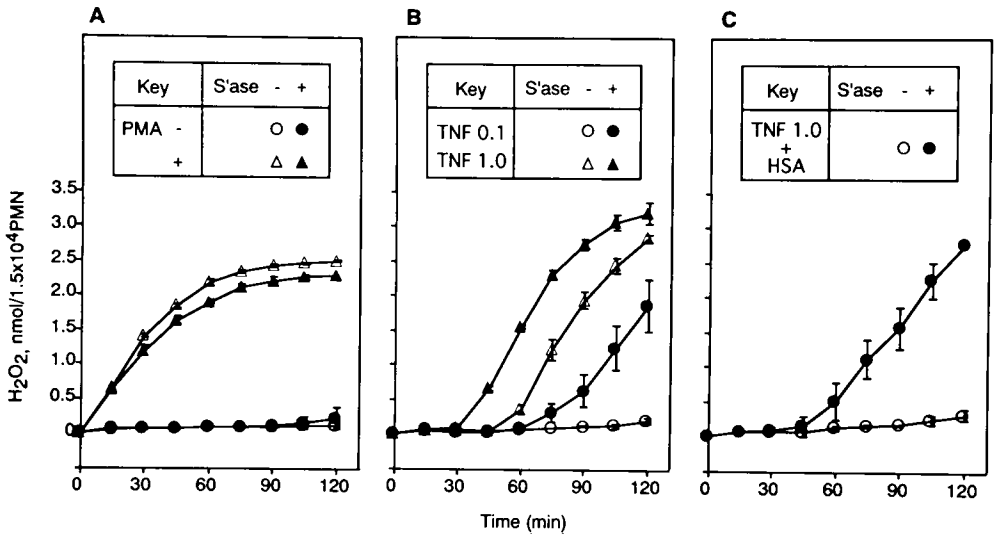

Figure 5. Effect of sialidase on the neutrophil respiratory burst. Neutrophils were exposed to *C. perfringens* sialidase (50 mU/ml for 30 min at 0°C) and then dispensed at 1.5 × 10^4 cells/ml to prewarmed, FBS-coated plates with (*A*) 0 or 100 ng/ml of PMA; (*B*) 0.1 or 1 ng/ml of TNF; or (*C*) 1 ng/ml of TNF with or without 100 μg/ml HSA. H_2O_2 release was monitored over the next 2 h. Data are means ± SEM of triplicates.

revealed a band at ~130-kD (17*a*). This was interpreted as leukosialin, yet it migrated faster than the material immunoprecipitated by a rabbit anti-leukosialin ab (17*a*). To resolve this issue, we surface-labeled PMN sialic acids as in the earlier studies (17*a*, 19), subjected the cell lysates to SDS-PAGE, compared the autoradiograms to immunoblots with anti-CD43 mAb, and tested the effect of pretreating the cells with sialidase (Fig. 6). The autoradiograms revealed a doublet at 125–137 kD, a relative molecular mass characteristic of CD43 in neutrophils (50). When periodate was omitted, no radiolabel was detected (not shown). Treatment of the neutrophils with 5 mU/ml *C. perfringens* sialidase for 20 min at 0°C before exposure to periodate reduced the intensity of radiolabeling and elevated the apparent molecular mass of the resulting species to 159 kD. With 10-fold more sialidase, a single radiolabeled species was faintly detectable at 175 kD (Fig. 6). The paradoxical decrease in mobility in SDS-PAGE after desialylation is characteristic of CD43 (50). Bands migrating at the same positions as on the autoradiograms, and no other bands, were seen when the same samples were immunoblotted with MEM59, a mAb specific for a sialyl-dependent epitope of CD43 (Fig. 6) (64). Thus, CD43 is the predominant sialoprotein on the surface of human neutrophils.

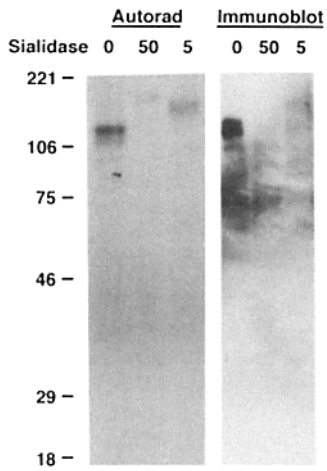

Figure 6. Characterization of sialoproteins on human PMN. Neutrophils were treated for 20 min at 0°C with 0, 5, or 50 mU/ml sialidase, followed by brief oxidation with $NaIO_4$ and reduction with ^{3}H-borohydride. The washed cell pellets were lysed and 100 μg cell protein/lane subjected to SDS-PAGE, followed by autoradiography or immunoblot with mAb MEM59, which detects a sialyl-dependent epitope of CD43. The migration of molecular mass markers is indicated.

HSA Partially Blocks Shedding of CD43 by TNF-Stimulated PMN

Since HSA and sialidase antagonized each other's effects on TNF-induced neutrophil responses, and CD43 was the predominant target of sialidase on neutrophils, we asked if HSA could block the shedding of CD43 from activated PMN. First, we confirmed that TNF (7, 55) and PMA (7, 32, 55) cause neutrophils to shed CD43, as monitored with L10 (Fig. 7), a mAb specific for a sialyl-independent epitope of CD43 contained within its 78 amino acid NH_2 terminus (51). While both suspended neutrophils and neutrophils adherent to FBS-coated plastic shed CD43 in response to PMA, only adherent cells shed CD43 in response to TNF (Fig. 7). These findings match the ability of PMA to stimulate a respiratory burst of similar magnitude in suspended and adherent PMN, while TNF only induces a respiratory burst in adherent cells (39).

The ~50% reduction in CD43 in adherent neutrophils stimulated with TNF was blocked completely by HSA, but not by a control protein, ovalbumin (Fig. 7). In contrast, HSA had no effect on the virtually complete shedding of CD43 induced by PMA (not shown).

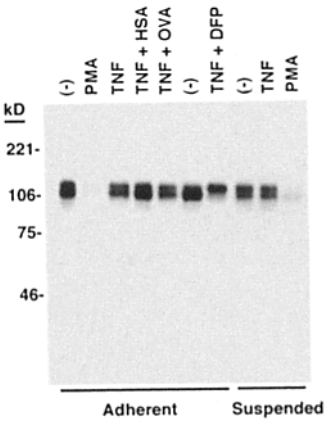

Figure 7. TNF- and PMA-induced shedding of CD43 from adherent or suspended neutrophils: effects of HSA and ovalbumin. Neutrophils (2 × 10^7/sample in 7.0 ml at 37°C) were tumbled in FBS-precoated tubes or allowed to adhere to FBS precoated Petri dishes for 60 min in the presence or absence of TNF (100 ng/ml), PMA (100 ng/ml), HSA (100 μg/ml), and/or ovalbumin (*OVA*) (100 μg/ml). Cells were collected as described in Materials and Methods, the washed pellets lysed, and 50 μg of cell protein/lane subjected to SDS-PAGE, followed by immunoblot with anti-CD43 mAb L10. The migration of molecular mass markers is indicated.

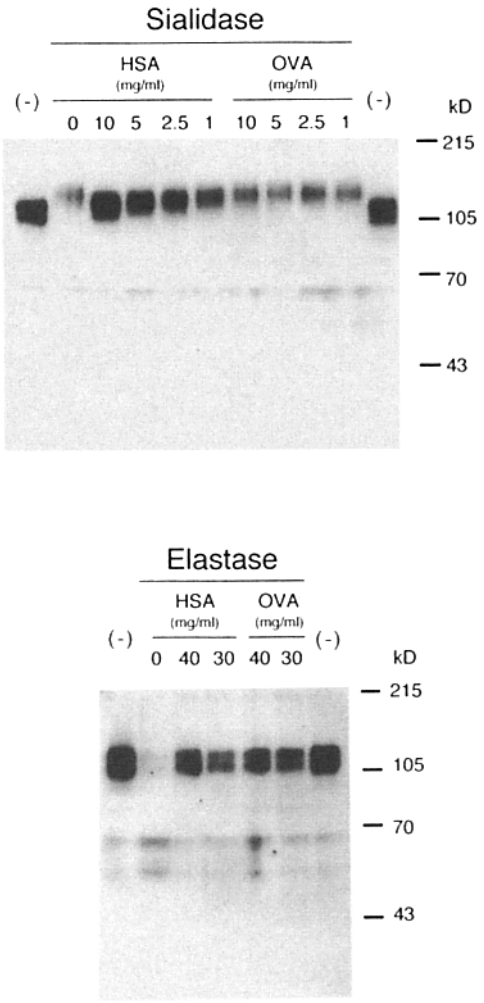

Figure 8. HSA blocks the actions of exogenous sialidase and elastase on neutrophil CD43. Neutrophils at 1.5 × 10^7 cells/ml were suspended for 10 min at 0° or 37°C with the indicated concentrations of HSA or ovalbumin (*OVA*), and then treated for 20 min further with 5 mU/ml sialidase (0°C) or 20 μg/ml neutrophil elastase (37°C). The washed cell pellets were lysed and 50 μg of cell protein/lane subjected to SDS-PAGE, followed by immunoblot with two anti-CD43 mAbs: (*upper panel*) MEM59, detecting a sialyl-dependent epitope, and (*lower panel*) L10, detecting a sialyl-independent epitope. The migration of molecular mass markers is indicated.

HSA Blocks the Actions of Exogenous Sialidase and Elastase on CD43

The ability of HSA to block shedding of CD43 by TNF-activated neutrophils might reflect protection of CD43 from endogenous sialidase and proteases. To test the plausibility of this hypothesis, we asked if HSA could protect CD43 from attack by exogenous enzymes.

In fact, the action of *C. perfringens* sialidase on neutrophil CD43 was completely blocked by HSA, but not by ovalbumin (Fig. 8). We reasoned that HSA could inhibit the action of sialidase on CD43 either by binding to sialidase, or by binding to CD43. To test the former possibility, *C. perfringens* sialidase was titrated against a synthetic fluorogenic substrate. Under reaction conditions linear with time and enzyme concentration, up to 10 mg/ml HSA inhibited hydrolysis of the sialidase substrate by no more than 12% (Fig. 9).

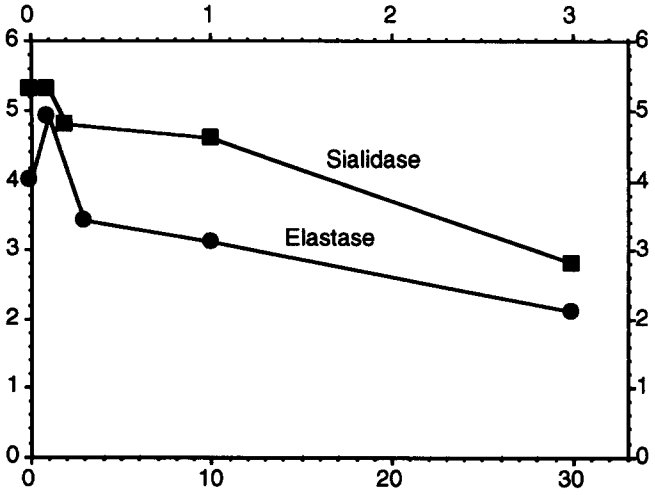

Figure 9. Effect of HSA on the hydrolysis of synthetic substrates by sialidase and elastase. *C. perfringens* sialidase was incubated with 4-methyl-umbelliferyl-*N*-acetyl-neuraminic acid and the concentrations of HSA (mg/ml) indicated on the lower abscissa (■). Purified neutrophil elastase was incubated with Ala-Ala-Pro-Val-7-amido-α-methyl-coumarin and the concentrations of HSA (mg/ml) indicated on the upper abscissa (●).

CD43 is generally protease-resistant (50, 51). However, pancreatic elastase can cleave CD43 from human lymphocytes in the presence of sialidase (51). Fig. 8 shows that neutrophil elastase can cleave CD43 from human neutrophils even without sialidase. When HSA was included in the reaction, cleavage of CD43 was inhibited. In contrast to the situation with sialidase, this was a nonspecific effect of HSA, in that high concentrations were required, ovalbumin had the same effect (Fig. 8), and as little as 3 mg/ml HSA inhibited by 48% the hydrolysis of a fluorogenic substrate by elastase (Fig. 9).

Thus, HSA blocked the action of elastase on CD43 by a mechanism that probably involved binding of HSA to elastase. In contrast, protection of CD43 from sialidase ap-

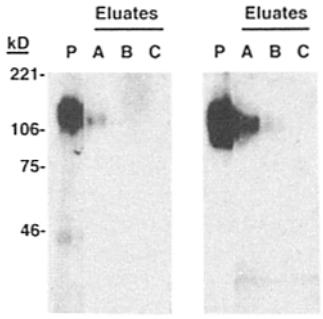

Figure 10. Affinity chromatography of shed extracellular domains of CD43 on HSA–Sepharose. Neutrophils were exposed to PMA (100 ng/ml) in the presence of protease inhibitors for 30 min at 37°C as described in Materials and Methods. One third of the supernatant was passed three times each over a column of CNBr-activated Sepharose to which had been coupled either (*A*) HSA, (*B*) ovalbumin, or (*C*) glycylglycine. The columns were washed extensively with KRPG and then eluted with 1 M NaCl. The eluates were concentrated on a membrane with a nominal 10-kD cut-off. Volumes deriving from the number of untreated neutrophils in lane *P* (intact PMN) (lane *P* contains 150 μg protein) were subjected to SDS-PAGE and immunoblotted with either of two anti-CD43 mAbs: (*left panel*) MEM59, detecting a sialyl-dependent epitope; (*right panel*) L10, detecting a sialyl-independent epitope. The migration of molecular mass markers is indicated.

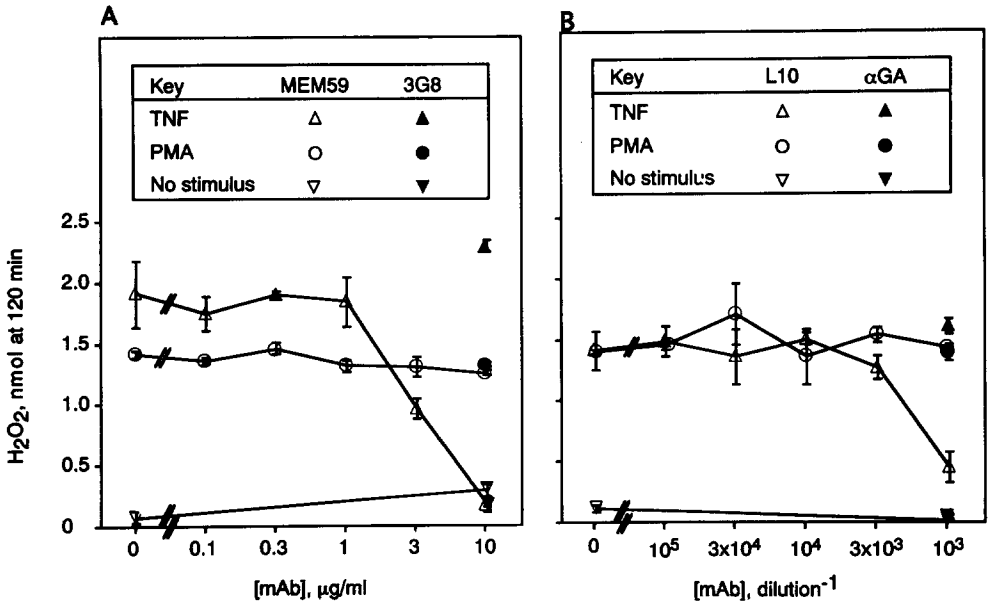

Figure 11. Inhibition of the TNF-induced respiratory burst by two anti-CD43 mAbs. 1.5×10^4 neutrophils were plated per well in FBS-precoated microtest plates containing the indicated concentrations of anti-CD43 or isotype-matched control mAbs. The cells were stimulated with TNF (100 ng/ml), PMA (100 ng/ml), or KRPG alone, and H_2O_2 release measured over the next 2 h by the fluorescent scopoletin assay. (*A*) MEM59 (*open symbols*) recognizes a sialyl-dependent epitope on CD43. The control mAb 3G8 (*solid symbols*) recognizes Fc receptor III, an abundant epitope on neutrophils (16). (*B*) L10 (*open symbols*) recognizes a sialyl-independent epitope on CD43. The control mAb αGA (*closed symbols*) recognizes gelsolin and is not expected to bind specifically to neutrophils. Data are means ± SE of triplicates.

peared to involve a specific interaction between HSA and CD43 itself.

The Extracellular Portion of CD43 Binds Specifically to HSA

The foregoing findings suggested that HSA may bind to CD43. Since HSA may bind to several different molecules on cell surfaces (21, 60) and this hypothesis does not require that CD43 be the major HSA binding site, we did not test the binding of HSA to cells, but instead tested the binding of CD43 to HSA. Since binding of the cytoplasmic and transmembrane domains of CD43 to HSA would lack physiologic relevance, and since detergents might interfere with binding, we avoided cell lysates, and instead used the extracellular portion of CD43. As a source, we suspended neutrophils in KRPG and stimulated them with PMA. We had to treat the cells with protease inhibitors to avoid degradation of shed CD43, even though this markedly decreased the amount of CD43 released (Nathan, C., and W. W. Jin, unpublished observations). The supernatant was passed over a column of HSA-Sepharose. The column was washed extensively with KRPG. Bound material was eluted with salt. As detected with mAbs MEM59 and L10, the eluate contained immunoreactive CD43, but only from the column to which HSA was coupled, and not from columns to which ovalbumin or glycylglycine were coupled (Fig. 10). The fragment of CD43 recovered in this manner migrated on SDS-PAGE much like holo-CD43. This matches observations with CD43 immunoprecipitated from the medium of PMA-treated neutrophils (32, 55). Thus, there is a specific interaction between HSA and the extracellular portion of CD43 in a medium whose ionic composition and pH reflect those of extracellular fluid.

Anti-CD43 mAbs Mimic the Inhibitory Action of HSA on TNF-stimulated PMN

If HSA inhibited TNF-induced cell spreading and the respiratory burst by binding to CD43, then other CD43-binding proteins might have the same effect. Indeed, two different anti-CD43 mAbs blocked the respiratory burst induced by TNF but not by PMA (Fig. 11). The mAbs were potent inhibitors (IC_{50} = 3 μg/ml for MEM59 IgG1 and 1:1,600 for L10 ascites). Likewise, both mAbs blocked cell spreading induced by TNF, but not by PMA (not shown). Two isotype-matched control mAbs, one of which was specific for an abundant epitope on neutrophils (16), were ineffective.

Discussion

Three sets of new findings emerge from these studies.

I. Selective Inhibition of Neutrophil Spreading by HSA

First, HSA can dissociate cell attachment from cell spreading. Thus, HSA did not interfere with agonist-stimulated adherence of neutrophils to protein-coated surfaces, but acted as a selective, reversible, nontoxic regulator of cell spreading induced by TNF, C5a or fMLF, and as an inhibitor of at least two of the metabolic responses that depend on spreading, the fall in cAMP and the respiratory burst. From this perspective, HSA can be classed with a structurally diverse group of secreted glycoproteins, such as osteonectin, tenascin and thrombospondin, that can support the attachment yet can antagonize the spreading of various cells (58).

Despite the widespread use of HSA or BSA in studies of neutrophil function, we know of no studies analyzing the effects of serum albumin on the metabolic and morphologic

behavior of neutrophils plated on surfaces coated with other proteins and stimulated with physiologic agonists. Nearly 30 years ago, Keller reported that HSA improved neutrophil function in chemotaxis chambers (25). He and other investigators went on to characterize HSA as a positive chemokinetic factor for neutrophils, that is, one which facilitates the cells' migration up a concentration gradient of another substance (26–28, 62, 67). One legacy of these reports is a view of albumin as a stimulus or tonic for neutrophils.

The present results suggest a different interpretation of albumin's chemokinetic effect: albumin inhibits extensive cell spreading, which compromises migration, while sustaining cell attachment, which facilitates it. This view is consistent with observations that albumin blunted the spreading of neutrophils on the polycarbonate membranes used in chemotaxis chambers (27, 28). The ability of HSA to dissociate cell attachment from cell spreading may make it an important physiologic regulator of motility.

Given the high concentration of albumin in human plasma (the middle of the normal range is 45 mg/ml), the question arises whether there can be any physiologic counterpart to the adhesion-dependent respiratory burst of neutrophils stimulated in vitro with soluble, physiologic agonists. In this context, it should be noted that suppression of the TNF-induced respiratory burst by HSA could be partially overridden by increasing the concentration of the agonist. Even at moderate TNF concentrations, suppression at the highest concentration of HSA that could be tested reached a plateau at 85%. This still left TNF-treated, adherent neutrophils releasing almost an order of magnitude more H_2O_2 than has typically been observed when neutrophils have been treated with soluble, physiologic agonists in suspension in the presence of cytochalasins (reviewed in ref. 39). Moreover, in early phases of inflammation, neutrophils can enter tissues before plasma proteins accumulate.

Thus, the impact of HSA on neutrophils is likely to be biphasic during the evolution of an inflammatory response. The asymmetric distribution of HSA at the outset (high intravascularly, low extravascularly) may favor egress of neutrophils from vessels and their retention in extravascular tissues, where they may be relatively free to spread, degranulate, and undergo a respiratory burst. Leveling of the *trans*-vascular gradient of HSA in later stages of inflammation may have the opposite effects.

On a weight basis, the potency of HSA (IC_{50} for H_2O_2 release, 46–180 μg/ml) and a subset of its CNBr fragments (IC_{50}, 18 μg/ml) was comparable with or greater than that of several peptides derived from extracellular matrix proteins that have been shown to antagonize cell spreading or adhesion in vitro. For example, 1 mg/ml of the fibrinogen-derived peptide GPRP decreased by ~50% the adherence of TNF-treated neutrophils to fibrinogen-coated surfaces (34). Similarly, spreading of BHK cells on a surface coated with a fragment of fibronectin was decreased by 50% by 1.1 mg/ml of GRGDS (1). Since these tetra- or pentapeptides (479 and 562 daltons, respectively) are far smaller than HSA (66.6 kD) or even the smallest of its CNBr fragments (3.4 kD), the potency of HSA and its bioactive fragments on a molar basis seems relatively high.

Inhibition of the TNF-triggered respiratory burst by plasma or serum could be accounted for quantitatively by their content of albumin (Nathan, C., unpublished observations). On the other hand, HSA is not the only plasma protein that can suppress TNF-induced spreading on matrix protein-coated surfaces and the associated respiratory burst. Fluid-phase fibronectin and fibrinogen can also do so (Nathan, C., unpublished observations), perhaps by binding to integrins and preventing them from clustering in focal adhesions. However, mimicry of soluble extracellular matrix proteins is unlikely to account for the mechanism of action of HSA as an antagonist of cell spreading. Although HSA shares 16 regions of primary sequence homology with fibronectin, vitronectin, von Willebrand factor, or the α, β, or γ chains of fibronectin, these regions are quite short, averaging 9.6 $\pm$ 0.6 (mean $\pm$ SEM) residues in length, within which amino acid identity with HSA averages only 5.4 $\pm$ 0.2 residues (56%) (Xie, Q.-W., and C. Nathan, unpublished observations). Neutrophil β2 integrins can bind to denatured proteins, including reduced and alkylated bovine serum albumin (13). This may have accounted for or contributed to the efficacy of certain CNBr fragments of HSA. However, there is no evidence that native HSA can bind to β2 integrins (13), and no reason to suspect that all the preparations of HSA used in our studies were more denatured than any of the control proteins. Finally, the lack of effect of HSA on TNF-induced adhesion of neutrophils to matrix protein-coated surfaces argues strongly against a primary mechanism of action based on binding to adhesion receptors. The experiments reported here support a different and apparently novel mechanism for the suppressive effects of HSA, as discussed below.

II. Regulation of Cell Spreading by CD43

The second conclusion of this work is that CD43 acts as an anti-spreading molecule on PMN. Thus, stimulus-induced spreading of neutrophils, and functional responses of the cells that depend on spreading, are closely linked to the ability of the cells to desialylate and/or shed CD43.

The 234 amino acids of the extracellular portion of human CD43 are subject to the addition of up to 85 O-linked carbohydrate chains (50), the number and nature of which depend on the type of cell and its state of differentiation (17). On rat T cells, CD43 has an extended conformation in which the extracellular domain protrudes 45 nm from the plasma membrane (11), farther than expected for any molecule on the surface of these cells. If this pertains to human PMN, on which CD43 evidently bears most of the sialic acid (Fig. 6), it is likely that CD43 mediates the first encounters a resting neutrophil makes with a supramolecular surface. The extraordinary length, negativity (pI = 4.1) and rigidity of CD43 (50) make it plausible that for integrins and other adhesion molecules to mediate the subsequent flattening of neutrophils against other cells or extracellular matrix, neutrophils must first shed, desialylate, and/or redistribute CD43 away from points of contact. On rat thymocytes and basophilic leukemia cells, CD43 colocalizes in microvilli with actin-associated proteins of the ezrin-radixin-moesin family and is redistributed into the cleavage furrow during mitosis (68). Similarly, CD43 on the neutrophil may redistribute to permit the rapid formation of limited intercellular contacts, before the cell commits to the more time-consuming and less readily reversible processes of trimming CD43 or cleaving it.

The physiologic functions of CD43 are unknown. On lym-

phocytes, CD43 can both mimic (57) and antagonize (3) the binding of lymphocyte function associated molecule-1 (LFA-1) to intercellular adhesion molecule-1 (ICAM-1, CD54). Cross-linking of CD43 on lymphocytes (46) and monocytes (44) can deliver an activation signal, but apparently not on neutrophils (29, 32). Functional deficits are evident in lymphoid and myeloid cells from patients with Wiskott-Aldrich syndrome, whose cells are CD43-deficient when studied ex vivo (50). These deficiencies include the chemotactic response of neutrophils to complement component C5a (45). However, the *CD43* gene is unaffected in Wiskott-Aldrich syndrome, and cell lines established from these patients express CD43 normally in vitro (38).

Normal human plasma contains >10 μg/ml of a mucin whose apparent molecular mass, carbohydrate composition and amino acid sequence match those of the extracellular domain of an isoform of CD43 that is expressed primarily on neutrophils and activated T lymphocytes (59). Since activated T cells are not known to shed CD43, neutrophils are the leading candidate for the origin of the soluble CD43 in the circulation. The functions of soluble CD43 are unknown.

Anti-CD43 antibodies circulate in many patients infected with HIV (2). If such antibodies act like the anti-CD43 mAbs studied here, they may make it difficult for HIV-infected people to mount a normal inflammatory response.

III. Mechanism of Action of HSA

Finally, we conclude that HSA's action as an antagonist of stimulus-induced spreading of neutrophils can be explained by its ability to bind to CD43 and diminish the susceptibility of CD43 to sialylytic and proteolytic attack. This represents a novel regulatory system of surprising complexity, in which a serum protein inhibits cell spreading by preventing cells from inactivating a cell-bound antagonist of spreading.

The notion that HSA can bind CD43 receives support from the recent characterization of a receptor used by microvascular endothelial cells for transcytosis of albumin (61). The gp60 albumin receptor is a glycoprotein bearing predominantly O-linked sialic acid residues (61). In these respects, gp60 resembles CD43. This resemblance, and the ability of sialidase to blunt the inhibitory effects of HSA on neutrophils (Fig. 5), suggest that HSA may bind to CD43 and gp60 via their sialyl groups. It is not clear what structural features of HSA (37) may confer this putative lectin activity. Nor is it known whether CD43 and gp60 are related at the level of their core polypeptides.

The endogenous enzymes responsible for removing CD43 from stimulated neutrophils have not been identified. Nor has it been explained why PMA caused more extensive shedding of CD43 than TNF, and did so in a manner resistant to inhibition by HSA. Three possibilities can be entertained.

First, PMA may elicit the release of the same CD43-degrading enzymes as TNF but in greater amounts, against which HSA may be relatively ineffective. Second, PMA may lead to the release of a different set of enzymes than TNF. Neutrophil elastase can cleave CD43 (Fig. 7), and the serine protease inhibitor DFP can partially antagonize TNF-induced shedding of CD43 (Fig. 7, and Nathan, C., and W. W. Jin, unpublished observations). Thus elastase or the related serine proteases cathepsin G and protease 3 (6) may account for a portion of the shedding elicited by TNF. These proteases may interact synergistically with endogenous sialidase (51). However, DFP did not block shedding induced by PMA. Thus, a DFP-resistant protease may be involved with both TNF and PMA as stimuli, playing a predominant role with PMA. Calpain released from sonicated platelets is a DFP-resistant protease that can specifically cleave desialylated CD43 from T lymphocytes (53), and neutrophils contain calpain (48). However, the apparent molecular mass of the cleavage product left behind by calpain (88 kD) (53) suggests that the released fragment could not exceed 62 kD. This is much smaller than the fragment recovered from the medium of PMA-activated neutrophils either by immunoprecipitation (32, 55) or by HSA-affinity chromatography (~125 kD; Fig. 10).

Finally, in human blood mononuclear cells and platelets, PMA induces hyperphosphorylation of serine residues in the cytoplasmic domain of CD43 (9, 47). Hyperphosphorylation of the cytoplasmic domain of CD43 might affect the intermolecular associations of the extracellular domains, for example, by preventing them from clustering with each other or with distinct molecules to which they normally bind, such as the Clq receptor (22). Altered intermolecular interactions of CD43 extracellular domains could enhance their susceptibility to hydrolases and/or diminish their ability to bind HSA.

Summary

Activation of neutrophils involves extensive remodeling of the cell surface with respect to molecules that affect adhesion and spreading. β2 integrins are brought forth (4, 29), while L-selectin (29) and CD43 (7, 32, 55; Fig. 7) are let go. How these processes are regulated and coordinated is not understood. The present findings suggest that adherence of neutrophils is a prerequisite for physiologic stimuli to induce shedding of CD43, while shedding of CD43 appears to be a prerequisite for neutrophils to spread and release large amounts of oxidants in response to the same stimuli. That HSA, the most abundant plasma protein, can suppress the shedding of CD43 suggests the importance to the host of controlling when, where and to what extent neutrophils spread.

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