Clin Appl Thrombosis/Hemostasis, 2(2):116–122, 1996

Anticoagulant Glycosaminoglycans Activate Respiratory Burst in Neutrophils and Monocytes

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Summary: Using four different glycosaminoglycans (unfractionated heparin, low-molecular-weight heparin, pentosan polysulfate, lactobionic acid) and the polypeptide hirudin, we investigated the influence of these anticoagulant substances on both polymorphonuclear neutrophils and monocytes respiratory burst in vitro. Using real-time analysis, we demonstrated that both unfractionated and low-molecular-weight heparins, as well as pentosan polysulfate, trigger the respiratory burst in neutrophils and monocytes within 3 to 5 s at clinically relevant concentrations. These effects could be antagonized by preincubating the anticoagulant drugs with protamine. Scanning electron microscopy confirmed an activated neutrophil phenotype after exposure to the anticoagulant compounds. Furthermore, we demonstrated that neither increased intracellular Ca^{2+} levels nor tyrosine phosphorylation of proteins, both mechanisms commonly involved in the signal transduction in neutrophils, participate in the glycosaminoglycan-mediated activation of the respiratory burst. Our results indicate that defined anticoagulant substances can trigger the respiratory burst in vitro, which might also have clinical implications in certain diseases. **Key Words:** Glycosaminoglycans—Hirudin—Polymorphonuclear leukocytes—Monocytes—Respiratory burst—Flow cytometry.

Heparin and other glycosaminoglycans (GAGs) are routinely used for prophylaxis and therapy of coagulation disorders. GAGs represent sulfated polysaccharides with multiple acidic groups, conferring a high negative charge on the molecule. Although ample data demonstrate that GAGs interfere with several soluble factors of the blood-clotting system [for a recent review see (1)], previous studies have provided evidence that these drugs might also bind to (2) and interfere with immune competent cells. It has been suggested that heparins influence the proliferative capacity of lymphocytes (3,4), and very recently it was demonstrated that heparin disaccharides and highly sulfated pentosan polysulfate (PPS) suppress natural killer cell activity (5,6). Furthermore, some reports demonstrate that heparins interfere with human polymorphonuclear neutrophil (PMN) function. These cells are known to eliminate invading microorganisms by uptake and degradation, and it has been suggested that these cells might also participate in such diseases as ischemia and adult respiratory distress syndrome by massive release of cytotoxic products (7,8). It has been shown that heparins increase both PMN aggregation and degranulation after costimulation with chemotactic factors (9), whereas others reported antiinflammatory properties of these saccharides. Besides reduced chemotactic activity (10), these authors also report decreased formylated Met-Leu-Phe (fMLP) or C5a-mediated granulocyte activation (11), suppressed phagocytosis of bacteria (12), and reduced fMLP-induced superoxide anion generation and chemiluminescence after stimulation with GAGs (11). The respiratory burst (RB) is one of the major components of acute inflammation, and it is involved in the killing and degradation of bacteria. It is an oxygen-dependent process, resulting in the production of superoxide, O_2^-, and H_2O_2. These substances are weakly microbicidal. However, H_2O_2 reacts with a chloride in the presence of myeloperoxidase to produce the powerful microbicidal agent, hyperchlorite. This H_2O_2–halide–myeloperoxidase system is the major antimicrobial system in the neutrophils [reviewed in (13)]. This study was done to investigate the influence of five different anticoagulant drugs on polymorphonuclear leukocytes in vitro at clinically relevant dosages. Using real-time analysis, we demonstrated

Manuscript received April 25, 1995; accepted May 1, 1995.
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that defined GAGs elicit the RB in both neutrophils and monocytes and show, by means of scanning electron microscopy, morphological alteration of GAG-activated neutrophils.

MATERIAL AND METHODS

Test substances

In accordance with concentrations used in the prophylaxis and therapy of thrombotic disorders, the following concentrations were applied in the assay (14): unfractionated heparin (UFH; Thrombophob; Nordmark, Ütersen, FRG): 0.1 U/ml, 1 U/ml, 10 U/ml; low-molecular-weight heparin (LMWH; Mono-Embolex; Sandoz, Nürnberg, FRG): 0.1 anti Xa-U/ml, 1 anti Xa-U/ml, 10 anti Xa-U/ml; pentosan polysulfate (PPS; SP 54; bene-Arzneimittel, Munich, FRG): 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml; lactobionic acid (LA; Aprosulat; Luitpold Werke, Munich, FRG): 0.5 ng/ml, 5 ng/ml, 50 ng/ml; and PEG-hirudin (Knoll-AG, Ludwigshafen, FRG): 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml. All test substances were diluted in Hank's buffered saline solutions (HBSS).

Preparation of leukocytes

Citrated whole blood was obtained from healthy volunteers and mixed with 0.5 volumes of dextran solution [1.5% Dextran-T-500 in phosphate-buffered saline (PBS), Pharmacia, Sweden]. Leukocyte-rich plasma was withdrawn, and erythrocytes were lysed twice by hypotonic shock for 50 s. After restoration of isotonicity, leukocytes were washed twice and adjusted to 1×10^6 PMN/ml in HBSS and stored on ice until used (15).

Separation of PMN

Polymorphonuclear leukocytes were separated from citrated whole blood by Ficoll-Hypaque gradient centrifugation (16) and erythrocytes were lysed with NH_4Cl. After three washings, cells were used as described previously. Murine PMNs were obtained from 6-week-old BALB/c mice by retroorbital plexus puncture and were isolated using Ficoll-Hypaque as described.

Respiratory burst

Cell suspension (1 ml) was incubated with 1 μl dihydrorhodamin (DHR) 123 (stock: 43.3 mM in DMF; Molecular Probes, Eugene, OR, U.S.A.) for 1–15 min at 37°C. Stained cells (50 μl) were mixed with the same volume of HBSS, fMLP, or one of the test substances. During the RB, the unpolar, nonfluorescent DHR123 is hydrolyzed, and the now green fluorescent rhodamin is trapped in the cells. The mean green fluorescence intensity of the PMN population was analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg) and used as a parameter for RB activity as described elsewhere (17).

Kinetics of RB activity

To investigate the onset of the RB after addition of GAGs, a real-time analysis assay was developed. To allow injection of the test substances during analysis, standard 12 mm × 75 mm fluorescence-activated cell scanner (FACS) tubes were punctured with a 3-mm electronic drill at the lower third of the tube, followed by sealing of the aperture by a silicone tube. A magnetic stirring bar and 1-ml cell suspension were placed in the tube and incubated for 10 min at 37°C. DHR was added (1 μl/ml), and the tubes were mounted on the flow cytometer. To keep the temperature at a constant level, a magnetic stirrer equipped with a heating device was placed directly beneath the tube. After addition of DHR (60 s) analysis was started, and after additional 50 s, 10 μl of the test substances was injected through the lateral aperture into the stirred sample using a Hamilton syringe. RB was monitored over a 5-min period.

Influence of protamine on RB

To investigate the influence of protamine (Protamine 1000/−5000; Roche; Hoffmann-La Roche, Grenzach-Wyhlen, FRG) on GAG-induced RB, protamine and GAGs were preincubated for 1 min and injected into the cell samples as outlined previously.

CD62L and PPS-mediated RB

To investigate whether CD62L is involved in GAG-mediated activation of the RB, murine PMNs were preincubated with anti-CD62L-monoclonal antibody (mAb) (5 μg/ml; Mel-14, Pharmingen, San Diego, CA, U.S.A.) loaded with DHR, and RB was analyzed in the presence of PPS (0.1 mg/ml).

Scanning electron microscopy (SEM)

Human PMN were incubated with PPS for 30 min, centrifuged to cover slides, and prepared for SEM by standard methods (18). Cells were fixed with 1% glutaraldehyde fixative solution for 16 h, dehydrated in graded alcohol, critical-end-point dried and gold coated. Scanning electron micrographs were taken at 5 kV and a magnification of 10,000 in a Jeol 6,300F scanning electron microscope (Kontron, Eching, FRG).

Cytoplasmic free calcium

Cytoplasmic free calcium was analyzed as described previously (19) using the fluorescent calcium indicator Fura-2 AM, which exhibits a calcium-dependent fluorescence at 505 nm after exci-

tation with 340 nm (Molecular Probes, Eugene, OR, U.S.A.). In brief, 10^7 cells/ml were loaded with 1 μM Fura-2 AM for 30 min at 37°C in PBS without Ca^{2+} or Mg^{2+}. Cells were washed three times with Hepes-buffered Krebs Henseleit buffer and placed in a spectrophotometer (F-3010, Hitachi, Berlin, FRG). Cells were kept at 37°C, and after 1 min, 10 μl of the test substances was added to 990 μl cell suspension. Fluorescence intensity at 505 nm was monitored up to 15 min.

Tyrosine phosphorylation

Tyrosine-phosphorylated proteins were detected as described elsewhere (20). Neutrophils (1.5×10^7/ml in HBSS) were incubated for 5 min at 37°C before stimulation with GAGs, hirudin, or interleukin-8 (IL-8, Gibco Life Technologies, Eggenstein, FRG). After various incubation periods (1–20 min), 100 μl of the cell suspension was mixed with the same volume of 2X Laemli's sample buffer preheated to 95°C. The samples were boiled for 7 min to denature the proteins completely and were then loaded onto 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 21). After electrophoretic transfer, tyrosine-phosphorylated proteins were revealed using the phosphotyrosine-specific mAb UB 05-321 (UBI, Lake Placid, NY, U.S.A.) at a final concentration of 1:4000. Phosphotyrosine bands were visualized using the enhanced chemiluminescence Western detection system (Amersham, Braunschweig, FRG).

Statistics

Statistical analysis was done using the Wilcoxon nonparametric test for matched samples.

RESULTS

RB after addition of GAGs or hirudin

DHR-labeled leukocytes were stimulated for 15 min with different GAGs, hirudin, or the bacteria-derived formylated tripeptide Met-Leu-Phe (fMLP) before flow-cytometric analysis. Using electronic gates, both PMN and monocytes were easily identified, and the mean DHR fluorescence of both cell populations was evaluated. Relative RB activity was determined by adjusting the mean channel value of the HBSS control sample to 1. Relative to this value, PMN DHR fluorescence increased 2.6-fold after stimulation with the positive control fMLP (2×10^{-6} M; Fig. 1A). Furthermore, UFH, LMWH, and PPS also showed a positive dose-dependent activation of the RB. Both UFH and LMWH triggered the RB significantly at the middle and high dosage tested (Fig. 1A), whereas PPS significantly triggered the RB at all concentrations tested, reaching values similar to those of fMLP at the high dosage (Fig. 1A). Neither the synthetic GAG LA nor the polypeptide hirudin showed any influence on neutrophil RB (Fig. 1A).

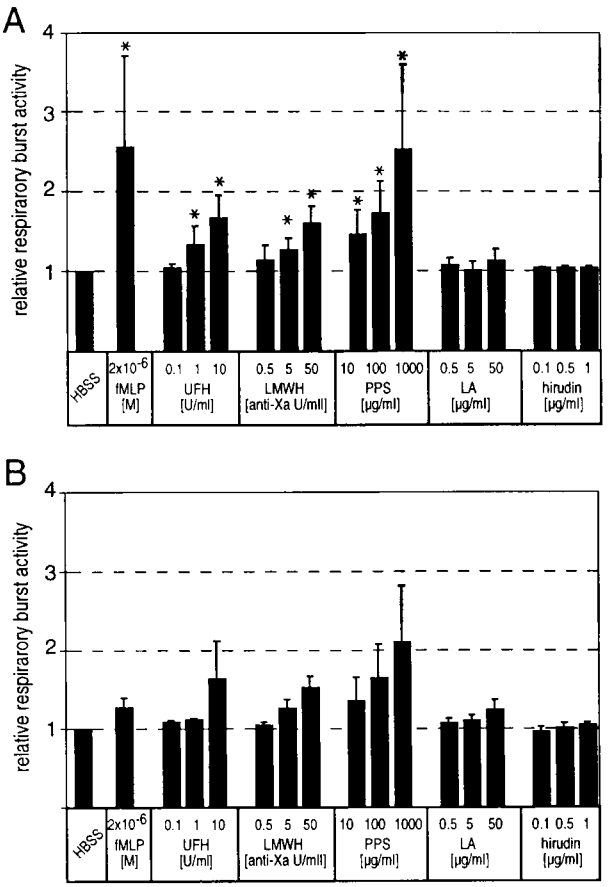

FIG. 1. Respiratory burst of polymorphonuclear neutrophils (PMNs) and monocytes. Leukocytes obtained after dextran sedimentation were labeled with dihydrorhodamin for 15 min before the substances were added as indicated. After a further 15 min, the mean green fluorescence intensities of the PMNs [**(A)** $n = 6$ + SD; $^*p < 0.05$] and monocytes [**(B)** $n = 3$ + SD, significance not tested] were analyzed. In both cases, respiratory burst values are expressed as multiples of unstimulated Hanks buffered saline solution (HBSS) control.

Using electronic gates, DHR-specific fluorescence intensity of the monocyte population could be analyzed in the same samples. As shown in Fig. 1B, the RB of monocytes was similarly activated by UFH, LMWH, and PPS, as shown for neutrophils, and again, neither LA nor hirudin showed any effect on monocyte RB.

Kinetics of GAG activated RB

To determine the onset of the GAG-induced RB, real-time analysis was conducted. As expected, the addition of HBSS did not lead to a change in DHR fluorescence intensity (Fig. 2A). In contrast, UFH, LMWH, or PPS triggered RB immediately within 3 to 5 s. As demonstrated previously, PPS triggered

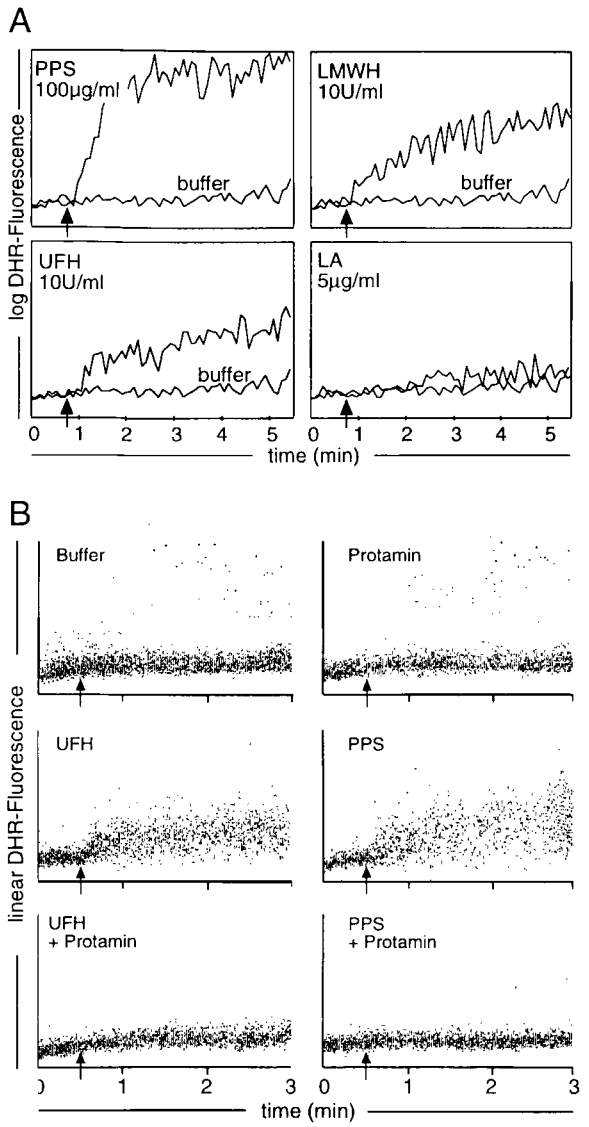

FIG. 2. Real-time analysis of the respiratory burst. Ficoll-separated polymorphonuclear neutrophils (PMNs) were labeled with dihydrorhodamin (DHR) for 1 min. Samples were analyzed by flow cytometry, and at the time points indicated, the substances were injected into the sample as indicated. **A:** mean log DHR fluorescence of the PMN; **B:** linear DHR fluorescence of single PMN.

RB stronger than UFH or LMWH, whereas LA did not show any effect (Fig. 2A).

Influence of protamine on GAG-activated RB

As protamine is clinically used to antagonize anticoagulant effects of GAGs (22,23) we tested whether this polycationic substance can suppress the GAG-induced RB as well. Preliminary experiments demonstrated that protamine does not interfere with cell viability at ≤1 U/ml and that this substance is able to antagonize the anticoagulant activity of UFH (0.1 U/ml) or PPS (0.1 mg/ml), each at 0.1 U/ml protamine in vitro (data not shown). Consequently, PPS or UFH was preincubated with protamine (0.1 U/ml) for 5 min before the mixture was added to DHR-labeled PMN. At this concentration, protamine suppressed the GAG-mediated RB of UFH or PPS as well (Fig. 2B).

Morphological changes of neutrophils after stimulation with PPS

PMNs were incubated with medium, PPS, or hirudin for 30 min before the cells were investigated by SEM. Untreated PMNs showed large and radially arranged microplicae and spacious microvilli. These are in close contact with each other, leading to the formation of caverns (Fig. 3A). In contrast, addition of PPS led to an expansive spread of the basal cytoplasm and reduced the expression of both microplicae and microvilli, resembling an activated status of the cell (Fig. 3B). Again, stimulation of PMNs with PEG-hirudin did not result in any detectable morphological modification (data not shown).

Effect of anti-CD62L mAb on PPS-induced RB

The adhesion molecule L-selectin (CD62L) is expressed on white blood cells and is involved in leukocytes' migration by mediating the attachment of these cells to high endothelial venules (24). It has been shown that heparin is a naturally occurring ligand for this molecule (25,26). As mAbs are available that neutralize the L-selectin–mediated cell adhesion in vitro and in vivo in the mouse (25,27), we used this mAb to investigate whether the adhesion molecule L-selectin is also involved in GAG-mediated activation of neutrophil RB. Murine PMNs were incubated with anti-CD62L mAb for 30 min and were subsequently stimulated with PPS. As shown in Fig. 4, preincubation of the anti-CD62L mAb did not antagonize GAG-induced RB, and no difference from CD62L-untreated samples could be observed. These data indicate that L-selectin is not involved in the GAG-mediated activation of the RB.

Cytosolic free Ca^{2+}

Increased free cytosolic Ca^{2+} is frequently observed after stimulating PMNs with various agonists like IL-8 and fMLP (28). To investigate whether increased free cytosolic Ca^{2+} is also involved in the signal transduction of GAG-mediated RB, we added IL-8 or the anticoagulant compounds to Fura-2 AM–loaded PMNs and monitored Ca^{2+}-specific fluorescence. As demonstrated for PPS, none of the GAGs gave rise to significant free intracellular calcium levels, whereas stimulation with IL-8 resulted in an immediate and strong Ca^{2+}-specific signal (Fig. 5A, data for UFH, LMWH, LA, and hirudin not shown). These results indicate that Ca^{2+} is not a second messenger in GAG-induced RB.

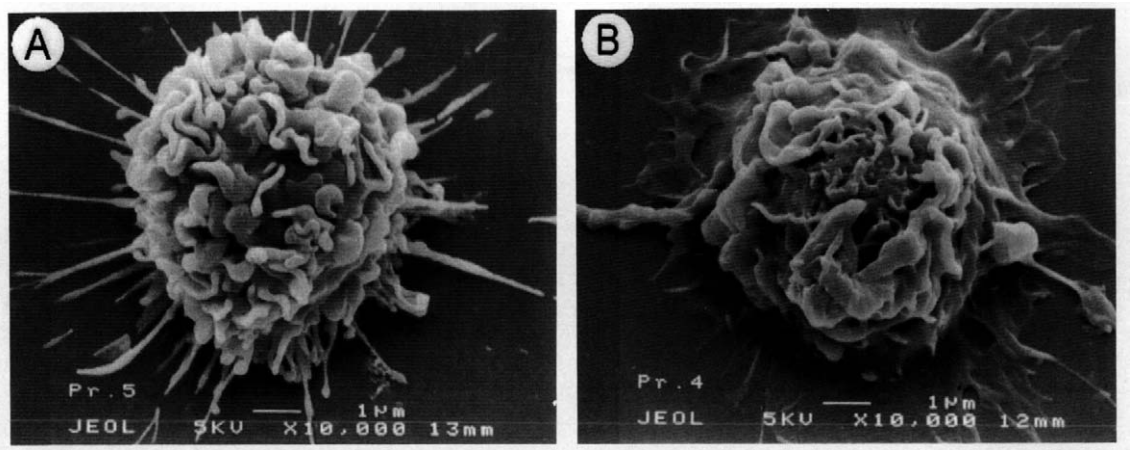

FIG. 3. Scanning electron microscopy of neutrophils after stimulation for 30 min with Hank's buffered saline solution (HBSS) **(A)** or 100 μg/ml pentosan polysulfate PPS **(B).**

Tyrosine phosphorylation

Recent studies have provided evidence that stimulation of neutrophils with various agonists like fMLP, IgG, granolocyte- or granulocyte/macrophage–colony-stimulating factor leads to tyrosine phosphorylation of several proteins (20,29–31). Therefore, IL-8 or PPS were incubated with PMNs for 1 to 20 min, and phosphorylation of tyrosine residues was determined by PAGE and Western blotting. As shown in Fig. 5B, stimulation of IL-8 causes tyrosine phosphorylation of proteins from 50 to 100 kDa. In contrast, the phosphotyrosine profile of PPS-stimulated PMNs did not differ from that of the unstimulated control. This result indicates that PPS-triggered RB is also not transduced by tyrosine-specific kinases.

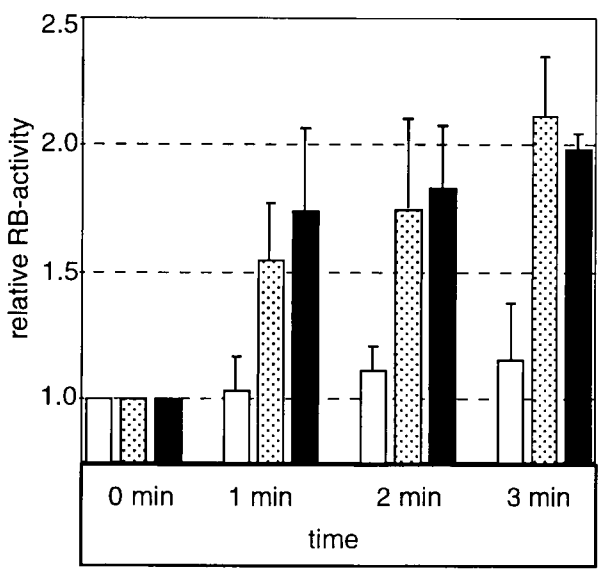

FIG. 4. Role of L-selectin. Murine neutrophils were labeled for 1 min with dihydrorhodamin and stimuli were injected at $t = 0$. At the time points indicated, relative respiratory-burst (RB) activity was analyzed. Open bars, Hank's buffered saline solution (HBSS); hatched bars, pentosan polysulfate (PPS; 100 μg/ml); black bars, anti-L-selectin–treated neutrophils (5 μg/ml, 30 min) stimulated with PPS (100 μg/ml).

DISCUSSION

Heparins and other GAGs are widely used for prophylaxis and treatment of thrombotic disorders. Depending on the chemical structure, these oligosaccharides interact with several components of the blood-clotting system [e.g., heparin-cofactor II, antithrombin III, tissue factor pathway inhibitor (TFPI)], and there is evidence that these molecules also bind to and interact with lymphatic cells (2,5). However, there is only limited information whether anticoagulant GAGs also interfere with monocyte or neutrophil function, and no data are available concerning the effects of hirudin on these cells. Phagocytosis of invasive microorganisms is the most characteristic feature of both cell types. Ingested particles are destroyed by the release of endogenous microbicidal enzymes and, even more important, by the production of several highly reactive oxygen metabolites (13,32). As the latter mechanism requires high amounts of oxygen, it is commonly referred as the RB. Several processes have been identified as leading to the activation of the RB, including ligation of cell-surface receptors for IgG for complement components, or for chemotactic substances, as well as for stimulation of neutrophils with phorbolesters or Ca^{2+} ionophores (33–36). As unimpaired neutrophils and monocytes are of paramount importance for the function of the immune system, we tested whether GAGs or hirudin functionally influence these cells at clinically relevant dosages.

Our study demonstrated that defined anticoagulant GAGs activate the RB of both neutrophils and

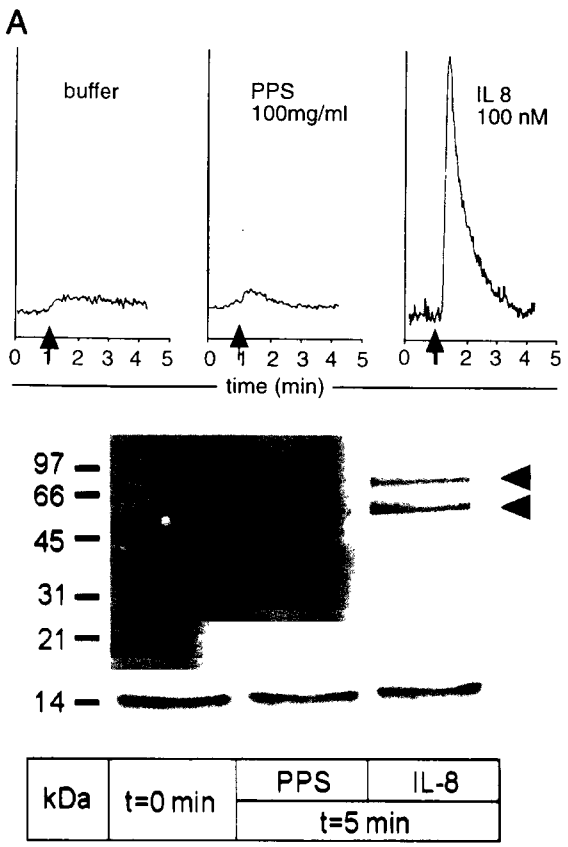

FIG. 5. Role of free cytoplasmatic Ca^{2+} and tyrosine phosphorylation. **A:** Human polymorphonuclear neutrophils (PMNs) were loaded with Fura-2, as described in Materials and Methods. At the time points indicated, substances were injected and Ca^{2+}-specific fluorescence was monitored at 505 nm. **B:** Before and after incubation with pentosan polysulfate (PPS; 100 μg/ml) or IL-8 (100 n*M*), PMNs were subjected to polyacrylamide gel electrophoresis (PAGE), and tyrosine-phosphorylated proteins were detected by Western blotting, using an anti-phosphotyrosine–specific monoclonal antibody as described in Materials and Methods.

monocytes, depending on the substance used and concentration applied (Fig. 1A and B). Incubation with PPS triggered the RB nearly to the same extent observed after activation with the bacteria-derived, chemotactic formylated tri-peptide fMLP, whereas incubation with UFH or LMWH resulted in less RB activation. In contrast, neither the anticoagulant polypeptide hirudin nor the synthetic GAG LA triggered the RB in neutrophils or monocytes (Fig. 1). By real-time analysis of the RB, we could show that the activation of the RB occurs immediately (3–5 s) after addition of PPS, LMWH, or UFH to neutrophils and reaches a plateau after ~1–2 min. (Fig. 2A). Preincubation of GAGs with protamine, a polycationic compound clinically used to antagonize the anticoagulant capacities of heparins, also neutralized their RB-promoting activities (Fig. 2B) but could not prevent suppression of natural killer cell activity reported earlier (6). Furthermore, by means of SEM, we could show that PPS-stimulated neutrophils exhibit a phenotype characteristic of activated phagocytes (Fig. 3B).

Our findings are in contrast to reports of others showing reduced cytochrome c reduction, lowered chemiluminescence, or reduced release of endogenous enzymes after incubation of PMNs with GAGs (11,14,37,38). However, in all these experiments, PMNs were pretreated with GAGs and after various periods were then subjected to several stimulatory substances like PMA, fMLP, or Ca^{2+} ionophores. Our results, however, provided evidence that GAGs induce the RB within a few seconds; because activation of the RB leads to exhaustion of the cells, it seems conceivable that GAG-activated neutrophils are less responsive to further stimuli.

Investigating the effect of GAGs on lymphocyte function, we and others earlier demonstrated (5,6) a suppressive effect of these compounds on natural killer cell activity, and as observed here for the respiratory burst, PPS showed the strongest effect. To identify neutrophil surface molecules involved in the binding of GAGs, we studied the effects of the adhesion molecule L-selectin, as it has been shown that heparin sulfate is the naturally occurring ligand for this molecule. However, the use of a mAb that has been shown to neutralize known effects of L-selectin in vivo and in vitro could not antagonize the GAG-mediated induction of the RB, and therefore, it seems unlikely that L-selectin is involved in this process (Fig. 4). For the identification of pathways by which the GAG-induced stimulatory signal is transduced into the cytoplasm of the cell, we looked for increased free cytoplasmatic Ca^{2+} levels and tyrosine phosphorylation of proteins. Both mechanisms frequently participate in the signal transduction after activation of neutrophil cell surface receptors (20,29–31,39). However, neither Ca^{2+} influx nor tyrosine phosphorylation of proteins seems to be involved in the activation of the RB with anticoagulant drugs (Fig. 5A and B), and further experiments must be done to identify signal-transducing molecules.

In conclusion, our data demonstrate that defined anticoagulant GAGs activate the RB in neutrophils and monocytes, which also might have clinical implications in various diseases associated with disseminated intravascular coagulation, septicemia, burns, obstetric accidents, and disseminated malignancy. Further clinical trials will show whether a stimulation of RB activity might be beneficial in critically ill patients.

Acknowledgment: We thank Dr. H.-G. Liebich and Mrs. E. Kuschek for helpful advice on scanning electron

microscopy and acknowledge the technical assistance of Mrs. C. Heinze and I. Eberl.

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