In Vitro Activation of the Contact (Hageman Factor) System of Plasma by Heparin and Chondroitin Sulfate E


A large number of negatively charged macromolecules, including DNA, glycosaminoglycans, and proteoglycans, were tested as possible activators of the contact (Hageman factor) system in vitro. Activation was assessed by conversion of prekallikrein to kallikrein, as determined by amidolytic assay and by cleavage of 125I-Hageman factor into 52,000- and 26,000-dalton fragments. Of particular interest to these studies, heparin proteoglycan and glycosaminoglycan from rat peritoneal mast cells, and squid chondroitin sulfate E, which is representative of the glycosaminoglycan from cultured mouse bone marrow derived mast cells, induced the reciprocal activation between Hageman factor and prekallikrein. In addition, naturally occurring heparin glycosaminoglycans from pig mucosa, bovine lung, and rat mast cells also induced activation. In contrast, native connective tissue matrix glycosaminoglycans and proteoglycans from several sources were inactive, although when one such chondroitin sulfate was further sulfated in vitro, it gained activity. When the negative charge of the activating agents was blocked by the addition of hexadimethrine bromide, the cleavage of 125I-Hageman factor in the presence of prekallikrein was prevented. The active negatively charged macromolecules induced cleavage of 125I-high molecular weight kininogen in normal plasma but not in Hageman factor-deficient or prekallikrein-deficient plasmas. Reconstitution of prekallikrein-deficient plasma with purified prekallikrein restored the kininogen cleavage upon addition of the active proteoglycans. These results suggest that both heparin from connective tissue mast cells and highly sulfated chondroitin sulfate E from cultured mouse bone marrow derived mast cells (which are considered synonymous with mucosal mast cells) could activate the contact system of plasma subsequent to an activation secretion response.

**MATERIALS AND METHODS**

**Materials**

Bz-Pro-Phe-Arg-pNA, Brij 35, pig mucosa heparin, human umbilical cord hyaluronic acid, and calf thymus deoxyribonucleic acid (Sigma Chemical Co., St. Louis, MO); D-Pro-Phe-Arg-pNA (Kabi Group Inc., Stockholm, Sweden); dextran sulfate (mol wt 500,000; sulfur content 17% ± 1%) (Pharmacia Fine Chemicals, Piscataway, NJ); bovine serum alumin (BSA) (Reheis Chemical Co., Kankakee, IL); x-ray films (XRP-l) (Eastman Kodak Co., Rochester, NY); whale cartilage chondroitin sulfate A, pig skin chondroitin sulfate B, and shark cartilage chondroitin sulfate C glycosaminoglycans (Miles Lab., Elkhart, IN) were obtained as noted. Human cartilage keratan sulfate, beef lung heparan sulfate, beef lung heparin, sturgeon notochord chondroitin sulfate A, pig mucosa chondroitin sulfate B, and sturgeon chondroitin sulfate C glycosaminoglycans were gifts from Dr. M. Mathews, University of Chicago. Squid chondroitin sulfate E was obtained from Dr. N. Seno, Ochanomizu University, Tokyo, Japan. Rat chondrosarcoma, pig cartilage, human intervertebral disc, and pig intervertebral disc monomer proteoglycans and proteoglycans aggregated to hyaluronate were extracted from the respective connective tissues under dissociative and associative conditions in the presence of protease inhibitors and were purified by published methods.

Rat serosal mast cells were obtained by the lavage of the peritoneal cavity of Sprague-Dawley rats and were concentrated to greater than 97% purity by isopycnic and isokinetic sedimentations.
Intracellular heparin proteoglycan was released from the mast cells by treatment with detergent-4 M guanidine-HCl and was purified by density gradient centrifugation. Serum heparin glycosaminoglycan was obtained by β-elimination of the chains from the native proteoglycan by treatment with 0.5 M NaOH. Chondroitin glycosaminoglycan, which was highly sulfated chemically to approximately 4 sulfate residues per disaccharide, was generously supplied by Dr. L. Goldman, Luitpold-Werk, Munich. Proteoglycan, approximately 4 sulfate residues, was obtained from normal individuals, and HF-deficient and PK-deficient (Fletcher) ACD plasmas were obtained from normal individuals, and HF-deficient and PK-deficient (Fletcher) ACD plasmas were obtained from characterized donors.

HF was isolated from fresh human plasma by the method of Griffin and Cochrane; its free amidase activity for D-Pro-Arg-pNA was less than 0.005% of that observed with an activated form. PK was purified from human plasma. Any kallikrein in the preparation (less than 0.5% of the total PK) was inactivated by treatment with 10 nM D-Pro-Arg-chloromethyl ketone, which was a gift from Drs. C. Kettner and E. Shaw, Brookhaven National Laboratories; the excess inhibitor was removed from PK by dialysis. Human plasma kallikrein (active form) was purified by the method of Nagase and Barrett. HMPW-K was purified by the method of Kerbiriou and Griffin, and 125I-HMPW-K (0.43 μCi/μg) and 125I-HF (10 μCi/μg) were prepared by the chloramine T method. All proteins used in this study were more than 90% homogeneous as assessed by SDS-polyacrylamide gel electrophoresis. Specific activity of HF was 80 unit/mg protein, where 1 U clotting activity is the amount of enzyme that produced almost 40% activation (Table 1). In the activation of the HF-PK mixture by glycosaminoglycans, and proteoglycan from pig mucosa, bovine nasal cartilage and type I collagen from guinea pig skin (acid soluble) were gifts from Dr. J. F. Woessner, University of Miami, and Dr. H. Hagase, Dartmouth Medical School, respectively. Acid citrate dextrose (ACD) plasmas were obtained from normal individuals, and HF-deficient and PK-deficient (Fletcher) ACD plasmas were obtained from characterized donors.

The reaction was stopped after a 5 min incubation by the addition of 500 μl of 2 N acetic acid.

Autoactivation of HF on NCS

Equal volumes of 40 μg/ml HF (in the above pH 5.3 buffer) and a solution containing NCS (10–2,000 μg/ml in 0.1 M Tris-HCl/0.02% NaN3, pH 7.5) were mixed and incubated at 25°C. At various incubation times, 20 μl of the mixture was withdrawn and added to 480 μl of 0.1 mM D-Pro-Phe-Arg-pNA (in 0.05 M Tris-HCl, pH 7.5) to determine the amidolytic activity of activated HF. After 5–60 min, the reaction was stopped by the addition of 500 μl of 2 N acetic acid.

Proteolytic Cleavage of 125I-HMPW-K in Normal and PK-Deficient Plasmas by Addition of Heparin or Dextran Sulfate

Three microliters of 0.1 M Tris-HCl (pH 7.4), 3 μl plasma, 1 μl 125I-HMPW-K (0.06 μCi, 0.14 μg), and 3 μl of the NCS (250 μg/ml, in saline) were mixed and incubated for 20 min in an ice bath. Twenty microliters of 10% SDS, containing 8 μg urea and 5% β-mercaptoethanol, was added, and the mixture was boiled for 4 min. A reconstitution experiment for PK was done by adding PK to PK-deficient plasma to a final concentration of 40 μg/ml. Samples were assayed on 9% polyacrylamide slab gels using the Laemmli system, followed by autoradiography with Kodak XRP-1 films.

RESULTS

Activation of the HF-PK Mixture by Glycosaminoglycan or Proteoglycan

To assess the capacity of various glycosaminoglycans and proteoglycans to stimulate the contact system, we combined various negatively charged substances (NCS) with purified HF and PK. The cofactor, HMPW-K, which would serve to increase the rate of such a reaction, is not essential and was therefore not included. In the activation system, which was composed of HF (10 μg/ml), PK (20 μg/ml), and an NCS (2.5–1,000 μg/ml) (all final concentrations), conversion of PK to kallikrein was observed to occur only when synthetic oversulfated chondroitin, or naturally occurring heparin glycosaminoglycans, and proteoglycan from pig mucosa, bovine lung, and rat mast cells were employed (Table 1). These NCS facilitated near-maximum activation at 12.5 μg/ml, and at this concentration, was equivalent to 5.0 μg/ml dextran sulfate. Squid chondroitin sulfate was slightly less active, but 12.5 μg/ml final concentration produced almost 40% activation (Table 1). In the absence of HF or NCS, the activation of PK did not take place (data not shown).

Upon activation with an NCS, HF in plasma has been shown to undergo proteolytic cleavage, yielding fragments of 52,000 and 28,000 daltons under reducing conditions. A similar cleavage is noted when purified HF and PK contact a negatively charged activating surface. Accordingly, 125I-HF (0.8 μg) was
added to 5 μg heparin from pig mucosa or 5 μg whale cartilage chondroitin sulfate A in the presence of 1.6 μg prekallikrein in 0.1 M Tris buffer, pH 7.5, in a total volume of 80 μl. After a 20-min incubation, an aliquot was removed from each mixture for amidolytic assay. When 10 μg hexadimethrine bromide was included, complete inhibition of cleavage of the HF resulted for 10 mm before addition of the other components. Complete inhibition of cleavage of the HF resulted when 10 μg hexadimethrine bromide was included, and prekallikrein activation was inhibited by 95%.

To determine if the various NCS could inhibit the detection of kallikrein, kallikrein was incubated with the various NCS and then assayed for amidase activity. Quantities of 12.5 and 250 μg/ml of NCS gave the following inhibition of 10 μg kallikrein: synthetic oversulfated chondroitin (8% and 40%), heparin glycosaminoglycans from pig mucosa (10% and 30%), bovine lung (24% and 45%), rat mast cells (0% and 8%), and heparin proteoglycan from rat peritoneal mast cells (11% and 32%). Dextran sulfate at concentrations of 1.25, 5, 12.5, 50, and 250 μg/ml inhibited 9%, 31%, 52%, 85%, and 95% of the kallikrein activity, respectively. Those substances not activating HF and PK did not inhibit kallikrein (<5%).

### Table 1. Activation of HF-PK on Various Glycosaminoglycans and Proteoglycans*

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*Calculated assuming that PK, 20 μg/ml, fully activated with β-HF, had the amidase activity of 0.26 U/ml.

†0% means 0%-0.9% activation.

### Proteolytic Cleavage of 125I-HMWK in Normal and PK-Deficient Plasma by Addition of Heparin or Dextran Sulfate

When normal plasma was mixed with rat mast cell heparin glycosaminoglycan or dextran sulfate at 75 μg/ml and incubated for 20 min at 0°C, cleavage of 125I-HMWK (mol wt 110,000) occurred, with subsequent generation of a 66,000 mol wt radiolabeled fragment (Fig. 1). PK-deficient plasma did not sustain this cleavage reaction (Fig. 2). However, if PK-
deficient plasma was reconstituted with PK using 40 
μg/ml plasma, the cleavage of 125I-HMWK was pro-
duced with the NCS (Fig. 2). Similar results were 
obtained with heparin glycosaminoglycans from pig 
mucosa and bovine lung and the native proteoglycan 
from rat mast cells, whereas none of the other glyco-
saminoglycans and proteoglycans facilitated the cleav-
ge of the 125I-HMWK.

Autoactivation of HF by NCS

When high concentrations of purified HF contact an 
NCS, a slow activation of the HF has been 
oberved.\textsuperscript{29,30} This has been termed “autoactivation” 
and represents strong evidence that the NCS has 
served as an activating surface. We therefore incu-
bated separately several NCS with purified HF to 
determine if the substance would fulfill this additional 
criterion of an activating surface. Figure 3 shows the 
time-course of autoactivation of HF (20 μg/ml) on 
heparin from pig mucosa (5–250 μg/ml) as deter-
mined by the appearance of amidase activity. The 
autoactivation was critically dependent on the concen-
tration of NCS added and the time of incubation. At a 
25 μg/ml heparin concentration, the rate of the 
appearance and the amount of amidase activity was 
maximal. After a 20-hr incubation, HF appeared to be 
fully activated and its specific activity was determined 
to be 7.8 U/mg. The optimal concentrations of syn-
thetic oversulfated chondroitin, heparin glycosamino-
glycans from bovine lung and rat mast cells, heparin 
proteoglycans from rat peritoneal mast cells, and pro-
teoglycan from rat peritoneal mast cells were 25, 25, 75, 
and 37.5 μg/ml, respectively. As shown in an insert 
to Fig. 3, autoactivation of HF with the optimum 
concentration (16 μg/ml) of dextran sulfate was faster. 
After a 5-min lag phase, the amidase activity 
rapidly appeared, and 30 min later, the activity 
reached a plateau with 7.5 U/mg HF.
Activation of Hageman factor (HF) and prekallikrein (PK) (Table 1) and cleavage of $^{125}$I-HMWK in plasma (Figs. 1 and 2) resulted from the addition of heparin glycosaminoglycans from rat mast cells, bovine lung, and pig mucosa, heparin proteoglycan from rat peritoneal mast cells, synthetic oversulfated chondroitin sulfate, squid chondroitin sulfate E (which is representative of the glycosaminoglycan of cultured mouse bone marrow derived mast cells), and dextran sulfate. The measured amidolytic activity to Bz-Pro-Phe-Arg-pNA was determined with 0.1 mM D-Pro-Phe-Arg-pNA at pH 7.5 and 25°C. Amidase activity was determined with 0.1 mM D-Pro-Phe-Arg-pNA at pH 7.5 and 25°C. Heparin concentrations: (x) 5 µg/ml; (♂) 25; (♀) 50; (△) 100; and (□) 250. Insert: with the optimum concentration of dextran sulfate, 16 µg/ml.

**DISCUSSION**

Activation of Hageman factor (HF) and prekallikrein (PK) (Table 1) and cleavage of $^{125}$I-HMWK in plasma (Figs. 1 and 2) resulted from the addition of heparin glycosaminoglycans from rat mast cells, bovine lung, and pig mucosa, heparin proteoglycan from rat peritoneal mast cells, synthetic oversulfated chondroitin sulfate, squid chondroitin sulfate E (which is representative of the glycosaminoglycan of cultured mouse bone marrow derived mast cells), and dextran sulfate. The measured amidolytic activity to Bz-Pro-Phe-Arg-pNA (0.26 U/ml) must be ascribed to activated PK, which was formed from the reciprocal activation between HF and PK in the presence of the negatively charged substance (NCS), as the reaction did not proceed in the absence of HF. That the contact system was activated was supported by the observation that HF underwent cleavage to 52,000- and 28,000-dalton subunits in the presence of pig mucosal heparin, and the cleavage of HF and activation of kallikrein was blocked by pretreatment of the heparin with hexadimethrine bromide. The ability to retain activity after $\beta$-elimination of the heparin chains from the native proteoglycan indicates that the larger 750,000 mol wt molecule is not required and that the interaction occurs through the 60–80,000 mol wt glycosaminoglycan chains.

Addition of several of the active substances to normal plasma induced the cleavage of $^{125}$I-HMWK molecules in the plasma, but not in HF-deficient and PK-deficient plasmas (Fig.1). However, when PK-deficient plasma was reconstituted with PK, cleavage of $^{125}$I-HMWK was induced (Fig. 2). Dextran sulfate showed the same effect on the cleavage of the radiolabeled protein, suggesting that addition of an active surface to plasma induces the activation of the contact system. None of the connective tissue derived native chondroitin sulfate glycosaminoglycans or chondroitin sulfate-containing proteoglycans was active.

The ability of the chemically sulfated chondroitin and naturally occurring heparin to activate the contact system suggests that the sugar linkage ($\beta$1–4 or $\beta$1–3), the hexuronic acid (glucuronic acid or iduronic acid), and the hexosamine (N-acetylgalactosamine or N-SO$_4$-glucosamine) are not of critical importance, as they differ in these two glycosaminoglycans. The presence of approximately four sulfate residues per disaccharide in synthetic oversulfated chondroitin and 2–3 sulfate residues per disaccharide in heparin or chondroitin sulfate E glycosaminoglycans, compared to 1–2 sulfate residues per disaccharide in the connective tissue derived natural chondroitin sulfate, suggests that activation is most strongly correlated with the total amount of sulfation. Whether naturally occurring oversulfated chondroitin sulfate, produced by endothelium, can serve to activate the contact system is unknown.

In experiments of HF autoactivation on a surface (Fig. 3), only those materials inducing activation of HF and PK caused autoactivation of HF. These active substances were oversulfated chondroitin, heparin from pig mucosa, bovine lung, and rat mast cells, proteoglycan from rat peritoneal mast cells, and dextran sulfate. Although the triggering mechanism of the autoactivation has not been completely clarified, we hypothesize that HF molecules bound to an active surface are converted to two-chain HF$_2$ (α-HF$_2$), which then function reciprocally with prekallikrein/kallikrein.

There is accumulating evidence that the anaphylactic response, mediated by mast cells, involves contact system proteins. In anaphylaxis in rabbits, consumption of activities of HF and clotting factors XI and IX have been noted, and the release of bradykinin has been recorded during anaphylaxis in guinea pigs and in rats. A release of a kallikrein, along with histamine, has been observed in vitro with antigen challenge of sensitized guinea pig lung fragments. In human beings, there are two reports showing a fall in clotting activity of HMWK in individuals with insect stings. In a study by Smith et al., two individuals allergic to insect (hymenoptera) stings showed a fall of 30% and 35% of normal levels of HMWK, whereas HF and PK levels remained unchanged; the assays were performed in plasma taken at the time of hypotensive
shock. In a patient analyzed 14 hr after a wasp sting that induced anaphylactic shock, a profound fall in coagulant titer of HMWK and PK was noted, and HF that induced anaphylactic shock, a profound fall in shock. In a patient analyzed 14 hr after a wasp sting were unchanged. In this latter study, the fall in HMWK and PK levels of plasma could not be attributed to demonstrable anticoagulant released in the blood during anaphylaxis. An explanation of the diminished levels of HMWK in the two studies and PK in the latter is not certain. Although levels of HF were unchanged, it should be noted that a small amount of HF, activated at the site where mast cells were stimulated, could generate considerable conversion of PK to kallikrein. This amplification has been observed experimentally. Thus, heparin or chondroitin sulfate E, released from the mast cells during anaphylaxis, could initiate this reaction sequence by providing a negatively charged surface for the binding of HF and, possibly, HMWK. Relative to this, an anticoagulant factor, indistinguishable from heparin, has been observed in allergic plasma that serves to bring about cleavage of $^{125}\text{I}}$-HF added to the plasma. This heparin-like factor was released into the arterial plasma of 4 asthmatic patients during challenge with aerosolized antigen during the development of bronchoconstriction. Alternatively, enzymes released from human lung and from isolated human lung mast cells by IgE-mediated reactions, have been shown to activate PK and to release bradykinin from HMWK. Mast cells isolated from human lung, upon stimulation with IgE and anti-IgE, were observed to release an enzyme capable of activating PK. Recently, intranasal challenge of allergic individuals with appropriate antigens induced release of bradykinin and lysylbradykinin, along with histamine and a TAME-esterase. It remains to be shown whether similar kinin-releasing systems are involved in these allergic reactions.

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In vitro activation of the contact (Hageman factor) system of plasma by heparin and chondroitin sulfate E

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