Evidence That the Serum Inhibitor of Hyaluronidase May Be a Member of the Inter-α-inhibitor Family*

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A study of the uncharacterized serum inhibitors of hyaluronidase, first described half a century ago, was undertaken. Activity was measured against bovine testicular hyaluronidase using a microtiter-based assay and reverse hyaluronan substrate gel zymography. The predominant inhibitory activity was magnesium-dependent and could be eliminated by protease or chondroitinase digestion and by heat treatment. Kinetics of inhibition were similar against hyaluronidases from testis and snake and bee venoms. The inhibitor had no effect on Streptomyces hyaluronidase, indicating that inhibition was not through protection of the hyaluronan substrate. Inhibition levels in serum were increased in mice following carbon tetrachloride or interleukin-1 injection, inducers of the acute-phase response. Reverse zymography identified a predominant band of 120-kDa relative molecular size, with two bands of greater and one of smaller size. The predominant protein was tentatively identified as a member of the inter-α-inhibitor family. Inhibition was also observed using either purified inter-α-inhibitor or an inter-α-inhibitor-related 120-kDa complex. Inter-α-inhibitor, found in the hyaluronan-rich cumulus mass surrounding mammalian ova and the coat of fibroblasts and mesothelial cells, may function to stabilize such matrices by protecting against hyaluronidase degradation. Turnover of circulating hyaluronan is extraordinarily rapid, with a half-life of 2–5 min. Prompt increases in levels of serum hyaluronan occur in patients with shock, septicemia, or massive burns, increases that can be attributed, in part, to suppression of degradation by these acute-phase reactants, the inhibitors of hyaluronidase.

Hyaluronan (HA) is a major component of the extracellular matrix, predominant whenever rapid cell proliferation, locomotion, and tissue repair occur (1–3). Hyaluronan is also involved in the organization and maintenance of matrix structures, as well as in water balance and electrolyte exchange (4). Levels of HA deposition are under exquisite controls that involve HA synthases (5–7), hyaluronidases (8–10), and growth factors that stimulate HA deposition (11, 12). The enzymes that digest HA, the hyaluronidases, are a family of proteins that, until recently, have been relatively neglected (8–10). Hyaluronidases play a key role in fertilization (13), embryonic development (14), wound healing (15), and neoplasia (16–18). Using molecular genetic techniques, it was established recently that there are seven hyaluronidases in the human genome, a paralogous group of six genes clustered three each on chromosomes 3p21.3 and 7q31 (19). The gene for a putative seventh hyaluronidase enzyme, unrelated to this group of six, is found on chromosome 10q24 (20).

Another level of control for HA deposition may be hyaluronidase inhibitors, a group of compounds about which little is known. The first studies documenting the existence of a circulatory inhibitor of hyaluronidase were published from 1946 to 1949 (21–23). These studies were followed by clinical reports that increased inhibitor levels occur in the sera of patients with cancer (24–26), liver disease (27), and dermatological disorders (28). These inhibitors were high molecular mass thermolabile glycoproteins (29) requiring magnesium ion for full activity (30). The inhibitor in cancer patients was qualitatively different, not requiring magnesium for activity (25). Despite the facts that evidence for such inhibitors has been available for over half a century and that they have obvious clinical importance, and even though a review appeared in 1955 that summarized these findings (31), no further characterization seems to have been undertaken.

Impediments to progress perhaps may be attributed to lack of sufficiently sensitive tools and to the absence of rapid assays that could facilitate detection of activity during the course of biochemical isolation. This is compounded by the fact that most serum and plasma proteins are large structures with multiple carbohydrate chains attached, making common techniques such as ion exchange and molecular sieve chromatography; isoelectric focusing; and sequential salt, alcohol, or pH precipitation and elution procedures notoriously difficult or ineffective.

The techniques that enabled our identification of the hyaluronidase family of enzymes were a microtiter-based assay (32) and a HA substrate gel zymography procedure (33). A variation of the latter was developed: reverse zymography in which the HA-containing gels are incubated in a solution of hyaluronidase. Stained bands mark the presence of inhibitors that protect the HA from degradation. Using this procedure, a major band of hyaluronidase inhibitor activity was observed in hu-
man plasma and serum specific for the neutral-active hyaluronidase associated with mammalian sperm, PH-20.

Recent studies show that a factor that induces compaction of follicular cells in oocyte cultures is a member of the inter-α-inhibitor (IαI) family present in fetal bovine serum added to culture medium (34–36). Inter-α-inhibitors are among the major Kunitz-type plasma protease inhibitors with large molecular sizes ranging from 130 to 240 kDa (37–39). The original isoform reported for IαI consists of two heavy chains and one light chain called bikunin. The three polypeptide chains are covalently linked by a chondroitin sulfate chain. Further studies verified the existence of a large range in molecular size of IαI molecules (40). In addition to the many genes coding for the heavy chains, divergence in the number and combination of peptides makes IαI a complex protein family.

In this study, the hypothesis that some of the circulating hyaluronidase inhibitors are members of the IαI family was examined. Microtiter-based enzyme inhibitor analyses, immunoprecipitation studies, and the reverse HA substrate gel procedure demonstrated that the molecular size of the predominant circulating hyaluronidase inhibitor is ~120 kDa and that it may be a member of the IαI family complex. Such inhibitors of hyaluronidase participating in the controls that establish levels of HA deposition in tissues may parallel the tissue inhibitor of metalloproteinases (41, 42).

**EXPERIMENTAL PROCEDURES**

**Materials**—Sera from various species were purchased from Sigma. Bovine testicular hyaluronidase (Wydase®) was kindly provided by Wyeth-Ayerst Co. (Philadelphia, PA). Covalink-NH microtiter plates were obtained from NUNC Co. (Placerville, NJ). Rabbit anti-human IαI antibodies and control rabbit IgG were purchased from Dako Corp. (Carpinteria, CA), and Streptomyces hyaluronidase was obtained from Calbiochem-Novabiochem. Human umbilical cord HA was the product of ICN Biomedical Inc. (Aurora, OH). Recombinant human plasma hyaluronidase-1 (Hyal-1) and monoclonal antibodies against Hyal-1 (17E9) were prepared in this laboratory as described (43). All other reagents were obtained from Sigma.

**Hyaluronan Substrate Gel—**Hyaluronidase assays were performed by a method reported previously (32). In brief, 1 mg/ml human umbilical cord HA in 0.1 mM Mes (pH 5.0) was biotinylated using 0.184 mg/ml N-hydroxysulfosuccinimide and 30 µM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The solution was dialyzed in distilled H2O and stored at −20 °C until used. The 96-well Covalink-NH plates were coated by placing 100 µl of diluted HA in 50 mM Mes (pH 5.0) and incubating at 4 °C overnight. The plates were washed with buffer and kept at 4 °C until used.

For the hyaluronidase assay, samples were diluted in a buffer solution containing 50 mM Hepes (pH 7.5), 0.1 mM NaCl, and 1% Triton X-100. Each sample of 100 µl was applied to the biotinylated HA-coated plate, and the plates were subjected to enzymatic reaction for 30 min at 37 °C. The amount of undigested biotinylated HA was measured using an avidin-biotin complex reaction, followed by o-phenylenediamine substrate color detection at 492 nm with an enzyme-linked immunosorbent assay plate reader (TiterTek Multiskan Plus, ICN Biomedical Inc.).

Hyaluronidase activity is expressed in relative turbidity reducing units (rTRUs) as defined previously (44). The data were analyzed using comparison with a set of standards generated by diluting the testicular hyaluronidase from 10 to 1 × 10−5 rTRUs/ml. The test enzyme activity was calculated to the amount of inhibitor added. Wydase® (bovine testicular hyaluronidase) and other neutral-active hyaluronidases were diluted to 1 rTRU/ml in buffer containing 50 mM Hepes (pH 7.5), 0.1 mM NaCl, and 1% Triton X-100. Mouse or human serum containing the putative hyaluronidase inhibitor activity was added to this solution at different concentrations (v/v). Suppression of testicular hyaluronidase activity was then measured.

**Hyaluronidase Inhibition Assay under Acid Conditions—**In the inhibition assay under acid conditions, Hyal-1-depleted human serum was used. It was necessary to eliminate endogenous acid-active hyaluronidase activity for this determination. Only mouse IgG antibodies against human Hyal-1 were available. For this reason, Hyal-1-immunodepleted human serum was used in this study, whereas all other studies were performed using mouse sera purified using an antibody capture technique. Equal volumes of 50% human serum in PBS with 17E9-conjugated beads were mixed. After rotation at 4 °C overnight, Hyal-1-depleted human serum was collected by centrifugation at 200 × g for 10 min to remove unbound antibodies. The complex was washed three times with 1 ml of PBS. Hyaluronidase in each fraction was assayed by adding 2% (v/v) serum to adjust for the level of inhibition activity. For experiments of pH dependence of inhibitor activity, Hyal-1-depleted human serum used as was a source of inhibitor.

**Hyaluronan Substrate Gel and Hyaluronidase Inhibitor Reverse HA Substrate Gel Procedures—**The HA substrate gel technique was applied to detect the hyaluronidase inhibitor. The HA substrate gel zymography technique, devised for the detection of hyaluronidase activity (33), was modified for the detection of hyaluronidase inhibitor activity.

The protocol described here was optimized for use with Bio-Rad 8 × 10-cm minigels. Samples were mixed with an equal volume of Laemmli buffer (45) and subjected to electrophoresis on 10% SDS-acrylamide gels containing 100 µg/ml HA. The SDS gels were then rinsed with 3% Triton X-100 for 1 h to substitute SDS, which may inactivate enzyme activity.

The gels were incubated at 37 °C for 16 h at 50 mM Hepes (pH 7.5), 0.15 M NaCl, and 1.0 mM MgCl2 containing 0.5 rTRU/ml bovine testicular hyaluronidase. The hyaluronidase digestion step can be difficult. The gel is fragile at this stage and must be handled with care. Minor changes in temperature and pH may produce variable results. For this reason, three levels of hyaluronidase were used with each experiment. 0.25, 0.50, and 2.00 rTRUs/ml were used to demonstrate that over- or under-digestion of the gels was not occurring.

Proteins in the gel, which may disturb penetration of dye into the gel, were digested by incubating gels in 0.1 mg/ml Pronase (containing 20 mg/ml Tris-HCl (pH 8.0)) for 4 h at 37 °C. Undigested HA was detected in the gels by Alcian blue staining. The gels were washed with 20% ethanol, 10% acetic acid, or 1% ethanol and 10% acetic acid for 20 min, followed by staining with 0.5% Alcian blue in 20% ethanol and 10% acetic acid for 1 h. This acidification of the Alcian blue enhances staining and prevents precipitation of dye. The gels were destained in 20% ethanol and 10% acetic acid until the inhibitor bands, representing undigested HA, appeared. This was accomplished in 1 h.

**Induction of the Acute-Phase Response by CCl4 and IL-1**—Two-month-old male Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA). CCl4 was mixed in olive oil at 1:3 (v/v) and injected into the peritoneum of mice (160 µl/20 g of body weight). After 6, 24, and 48 h, blood was collected from mice under CO2 anesthesia. Serum fractions were collected by centrifugation at 10,000 × g for 20 min following clot formation. Samples were kept frozen until used. IL-1, a kind gift from Dr. Charles Dinarello (University of Colorado Health Science Center, Denver, CO), was administered intraperitoneally (1 µg in 200 µl of PBS).

**Acidification and Digestion with the Chondroitinase Activity Associated with Plasma Hyaluronidase—**For the serum acidification studies, serum samples were mixed with an equal volume of either 100 mM Tris-HCl (pH 7.4) or 100 mM formate buffer (pH 3.7) and incubated at 37 °C. Samples were collected at each time period, frozen immediately, and stored until assayed.

**Chondroitinase Digestion—**Enzymatic digestion with bacterial chondroitinase was also performed. Chondroitinase AC (Flavobacterium heparinum; Sigma C-2780) was added to 50 µl of serum at 50 units/ml and incubated at 37 °C overnight. The reverse HA substrate gel zymography procedure was then performed as described above.
Immunoprecipitation Using Anti-inter-a-inhibitor Antibodies—The multiple immunoprecipitation step study was performed using small volumes of rabbit anti-human Ia1 antibodies. The purified immunoglobulin fraction (Dako A301) and control rabbit IgG (Dako X0903) were utilized in a protocol slightly modified from that described in a previous report (35). First, 7.6 mg of rabbit anti-human Ia1 antibodies or control rabbit IgG were conjugated with 0.5 ml of protein A-agarose by mixing at 4 °C overnight. The IgG-protein A-agarose complex was washed three times with PBS to eliminate unbound IgG. The volume of each IgG-protein A-agarose complex was adjusted to 0.5 ml. The complex was added to serum samples at 10% (v/v) and incubated at 4 °C for 2 h. Samples were centrifuged at 200 × g for 10 min to remove the pellets. This procedure was repeated four times. Hyaluronidase inhibition efficiency of each precipitation step in samples was compared with that of the untreated serum.

Hyaluronidase inhibition activity from precipitated beads was also examined using these antibodies. Rabbit anti-human Ia1 antibodies or control rabbit IgG was added to 50% mouse serum at final concentrations of 0.5, 1.25, and 2.5 mg/ml. Samples were incubated at 4 °C overnight and then centrifuged at 200 × g for 10 min to separate the supernatant and IgG-protein A-agarose complex. The IgG-protein A-agarose complex was washed three times with 1 ml of PBS. Hyaluronidase inhibition of the supernatant and IgG-protein A-agarose complex was measured.

Purification of Mouse Inter-a-inhibitor—Mouse Ia1 was isolated from mouse plasma according to the protocol described by Carrette et al. (46). The protocol was slightly modified to adapt it to the Akta™ purifier system (Amersham Pharmacia Biotech). Briefly, 10 ml of mouse plasma (RJO Biologicals, Inc., Kansas City, MO) were diluted 1:10 (v/v) in 20 mM Tris-HCl (pH 6.8) containing 0.3 mM NaCl and 4 mM EDTA (buffer A). The plasma was loaded onto a MonoQ HR5/5 column equilibrated in buffer A at a flow rate of 1 ml/min. After washing the column to remove unbound proteins, elution was performed by increasing the NaCl concentration to 0.6 M and then to 1 M. Ia1 was found in the fraction that eluted at 0.6 M NaCl. The fractions recovered from five different consecutive Q-Sepharose chromatography procedures were pooled and equilibrated by dialysis in 20 mM Tris-HCl (pH 6.8) containing 4 mM EDTA and 0.1 M NaCl. After concentration by ultrafiltration to 5 ml, the dialyzed sample was loaded onto a 5-ml HiTrap heparin-Sepharose column. Elution was performed at a flow rate of 2 ml/min using a 0.1–0.5 M NaCl linear gradient (total volume of 50 ml). An Ia1-related 120-kDa complex was found in a fraction that eluted at 0.35 M NaCl, and mouse Ia1 was recovered in a fraction that eluted at 0.4 M NaCl. The Ia1 fraction was ~95% free of other proteins as judged by Coomassie Blue staining following SDS-polyacrylamide gel electrophoresis. The purity of the Ia1-related 120-kDa product, however, was ~80%. Because of the high susceptibility of Ia1 to proteolysis, a mixture of protease inhibitors (Complete™, one tablet dissolved in 2 ml of water; Roche Molecular Biochemicals) was added at a 1% (v/v) final concentration to both samples for long-term storage before testing for hyaluronidase inhibitory activity.

RESULTS

Hyaluronidase inhibition activity was measured in sera using the enzyme-linked immunosorbent assay-like assay system. In preliminary data, mouse serum had approximately twice the levels of inhibitory activity as human serum. Mouse serum was therefore used in most subsequent experiments.

Levels of inhibition activity were examined in mouse serum using bovine testicular hyaluronidase as the test enzyme. This is a neutral-active hyaluronidase with a bimodal curve of pH optima at 4.5 and 7.5, possibly the result of two forms of the enzyme present in the preparation, a soluble and a membrane-bound form (47–49). The inhibition assay was performed at pH 7.5. Serum was examined between 0.25 and 2.0% (v/v) reaction mixture. The hyaluronidase activity was suppressed by addition of mouse serum in a dose-dependent manner. 50% inhibition was obtained at ~0.35% volume (Fig. 1).

Heparin, a well characterized inhibitor of hyaluronidase (50, 51), was also examined. 50% inhibition was obtained at a concentration of 0.2 mg/ml in the assay incubation mixture, which is far greater than physiological concentrations. The concentration of naturally occurring heparin in plasma is too low to be detected by conventional means.
serviced between pH 6 and 8 (Fig. 3), in sharp contrast to the wide range of activity reported for the test enzyme, bovine testicular hyaluronidase (43, 52, 53).

The serum inhibitory activity against the bovine enzyme was compared with other neutral-active hyaluronidases. Several microorganisms as well as the venom from a number of insects and species of snakes contain hyaluronidases active at neutral pH. Snake venom from the timber rattlesnake (Crotalus horridus horridus), bee venom from the honeybee (Apis mellifera), and hyaluronidase from Streptomyces hyaluronyticus were examined. The serum inhibitor had potent inhibitory activity against bovine testicular hyaluronidase, with 80% inhibition being observed in this experiment. The snake venom enzyme was inhibited 45%, and the bee venom hyaluronidase was inhibited 70%. No inhibition was observed against Streptomyces hyaluronidase, and only 15% inhibition was observed against recombinant acid-active human plasma Hyal-1 under these assay conditions (Fig. 4).

Heparin exerted a different profile of inhibition against these enzymes. Heparin inhibited snake venom hyaluronidase more efficiently than bovine testicular hyaluronidase. As with serum, heparin had no inhibitory effect on Streptomyces hyaluronidase, but exerted a major inhibitory effect on recombinant human Hyal-1 (Fig. 4).

A curious observation made in the course of these studies was that plasma contained far less inhibitory activity than did serum. To address this question, the effect of various cations was examined. Cations that may have been removed by the chelating agents used as anticoagulants in the preparation of plasma. The chloride forms of Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Hg$^{2+}$, and Cu$^{2+}$ at 2.0 mM were added to the hyaluronidase solution to which 0.5% of mouse plasma was added in advance. Recovery of inhibitor activity was examined. Addition of 0.5% plasma had no inhibitory activity against testicular hyaluronidase compared with the 48% inhibition by the same concentration of serum. At 2.0 mM Mg$^{2+}$, 70% of the inhibitory activity was recovered, comparable to the 80% inhibition observed when 0.5% serum was tested with 2.0 mM Mg$^{2+}$. No other cationic ions examined possessed the ability to reactivate inhibitor activity. Data for Zn$^{2+}$ and Fe$^{2+}$ could not be obtained because of the suppression of hyaluronidase activity that occurred in their presence.

These results were supported by additional experiments in which EDTA was added to serum to suppress activity (Table I). In the experiment shown in Table I, 0.5% (v/v) serum had a 48% level of inhibition, a 36% level in the presence of 0.2 mM EDTA, and no inhibitory activity with 2.0 mM EDTA. Addition of 2.0 mM MgCl$_2$ gave 79% inhibition.

The reverse HA substrate gel zymography procedure was applied in an attempt to identify the inhibitor molecules. Mouse serum was electrophoresed on SDS-polyacrylamide gel containing 100 μg/ml HA. The entire HA-containing gel was subjected to enzymatic digestion using a solution of bovine testicular hyaluronidase containing 1 mM Mg$^{2+}$. The undigested HA was detected by staining with Alcian blue, with the stained band corresponding to inhibitory activity. Two major bands of −120- and 90-kDa relative molecular size were observed. Minor bands were also observed of −190, 220, and 75 kDa.

To avoid under- and over-digestion of HA in the gel, three gels were prepared for each experiment using three different concentrations of hyaluronidase (0.25, 0.5, and 2.0 rTRUs/ml). Digestion and processing of the three gels were performed in parallel. As shown in Fig. 5, two predominant bands were observed in serum at 1, 2, and 4 μl (lanes 2–4, respectively). One band, however, corresponded to a major glycoprotein in serum that could be stained with Alcian blue on gels that did
TABLE I  
Modulation of serum hyaluronidase inhibition activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>48</td>
</tr>
<tr>
<td>EDTA 0.2 mM</td>
<td>36</td>
</tr>
<tr>
<td>EDTA 2.0 mM</td>
<td>0</td>
</tr>
<tr>
<td>MgCl₂ (2.0 mM)</td>
<td>79</td>
</tr>
</tbody>
</table>

Mouse serum was added at 0.5% (v/v) to 1 rTRU/ml bovine testicular hyaluronidase in 50 mM Hepes (pH 7.5), 0.1 M NaCl, and 1% Triton X-100. The indicated addition was then made. Values represent the average of two determinations. Differences between values was routinely <10%.

FIG. 5. Detection of hyaluronidase inhibitor activity using reverse HA substrate gel zymography. Serial dilutions of mouse serum were placed on SDS gel. Lane 1, protein standards. Lanes 2–4, detection of bands of the hyaluronidase inhibitor. Samples were placed on SDS gel containing 100 µg/ml HA. After incubation in 3% Triton-X to replace the SDS, the gel was subjected to enzymatic digestion by incubating in 0.5 rTRU/ml Wydase (bovine testicular hyaluronidase) solution (pH 7.5) at 37 °C overnight. Undigested HA was visualized by Alcian blue staining. Lanes 5–7, endogenous glycoprotein in serum. Samples were run using the same procedure described above, except that the gels did not contain HA. Lane 8, detection of hyaluronidase by the conventional HA substrate gel procedure at pH 3.7. Sample volumes in lanes 2, 5, and 8 were 1 µl of serum; in lanes 3 and 6, 2 µl of serum; and in lanes 4 and 7, 4 µl of serum. Closed arrow, the band of the hyaluronidase inhibitor; open arrows, plasma glycoprotein; closed star, human Hyal-1.

not contain HA (lanes 5–7). In all aspects, the samples were processed in the same way as those shown in the HA-containing gels. Thus, only one major band, or possibly several bands running close to each other at ~120 kDa, could be attributed to the serum hyaluronidase inhibitor. Lane 8 shows the position of mouse plasma hyaluronidase at 57 kDa in a gel run at pH 3.7.

A series of immunochemical experiments were performed to test the hypothesis that the predominant serum hyaluronidase inhibitor is a member of the IaI family. The commercially available anti-IaI antibodies used recognize all three heavy chains of the IaI family and bikunin. Serum was subjected to four sequential immunoprecipitation steps using small volumes of the anti-IaI antibody-conjugated beads. Hyaluronidase inhibition activities in serum supernatants were examined following each precipitation step. The same concentration of rabbit IgG was used as a control in the sequential steps. Serum lost increasing amounts of hyaluronidase inhibitor activity with each immunoprecipitation, whereas control rabbit IgG immunoprecipitation showed little loss of inhibitory activity (Fig. 6A). Further studies were performed to examine the inhibitory activity in the precipitated beads. In contrast to the decrease in inhibition activity in serum following immunoprecipitation, precipitated IaI beads gained inhibitory activity (Fig. 6B).

Immunodepletion was investigated further. Loss of hyaluronidase inhibitory activity was measured in mouse and human sera that had been immunodepleted of IaI using increasing amounts of anti-IaI antibodies. As shown in Table II (part A), mouse serum depleted of IaI by 1.25 and 2.50 mg/ml anti-IaI antibody had a loss of 20 and 39% of neutral hyaluronidase inhibitor activity, respectively, in the standard assay. Human serum depleted of IaI by 0.5 and 1.25 mg/ml anti-IaI antibody lost 52 and 69% of inhibitory activity, respectively. The effect on acid-active human Hyal-1 activity was far less effective (Table II, part B).

The IaI protein family consists of two or more peptide chains covalently linked by chondroitin sulfate (54, 55). Sensitivity to chondroitinase digestion is predicted to be a feature of the IaI family. Serum contains an endogenous hyaluronidase activity termed Hyal-1 that is an acid-active enzyme. Vertebrate hyaluronidases also digest chondroitin sulfate, albeit at a slower rate. Serum was acidified to pH 3.7 and incubated at 37 °C for 1 h to permit the chondroitinase reaction to occur. Serum was then re-neutralized using Hepes (pH 7.5), and hyaluronidase inhibition activity was measured. The inhibition activity was eliminated from serum samples that had been incubated at pH...
samples were incubated at pH 3.7 and then re-neutralized (Fig. 7). Of serum hyaluronidase inhibition activity was examined after acidification using enzyme-linked immunosorbent assay. Mouse sera were mixed with equal volumes of 100 mM Hepes (pH 7.5) or 100 mM formate buffer (pH 3.7) and incubated at pH 7.4. Mouse sera, after 18 h of incubation at pH 7.5, after incubation at pH 7.5, followed by re-neutralization; lane 5; after 18 h of incubation at pH 7.5; lane 6; after 18 h of incubation at pH 3.7, followed by re-neutralization; lane 17; after 18 h of incubation with 50 units/ml chondroitinase AC (F. heparinum) pH 7.5; lane 8, control sample incubation without the chondroitinase AC enzyme. Samples were applied to the SDS gel using 2 μl of serum/lane.

**Fig. 7.** Loss of hyaluronidase inhibition activity following serum acidification using enzyme-linked immunosorbent assay. Levels of serum hyaluronidase inhibition activity were examined after samples were incubated at pH 3.7 and then re-neutralized (○) or incubated at pH 7.4 (□). Mouse sera were mixed with equal volumes of 100 mM Hepes (pH 7.5) or 100 mM formate buffer (pH 3.7) and incubated at 37 °C for the indicated periods of time. Hyaluronidase inhibition activity was measured at 1% (w/v) serum using 1 rTRU/ml bovine testicular hyaluronidase.

**Fig. 8.** Loss of inhibitor activity following serum acidification or digestion with chondroitinase AC using reverse zymography procedure. Lane 1, protein standards; lane 2, band of hyaluronidase inhibition in untreated mouse serum; lane 3, after 2 h of incubation at pH 7.5; lane 4, after incubation at pH 7.5, followed by re-neutralization; lane 5; after 18 h of incubation at pH 7.5; lane 6; after 18 h of incubation at pH 3.7, followed by re-neutralization; lane 7; after 18 h of incubation with 50 units/ml chondroitinase AC (F. heparinum) pH 7.5; lane 8, control sample incubation without the chondroitinase AC enzyme. Samples were applied to the SDS gel using 2 μl of serum/lane.

3.7. Loss of activity was time-dependent during the incubation at pH 3.7 and was complete after 60 min (Fig. 7). The chondroitinase digestion-generated loss of hyaluronidase inhibition activity was confirmed by reverse HA substrate gel zymography. As shown in Fig. 8, bands of inhibition were lost in acidified and re-neutralized serum during the reverse zymography procedure. The predominant band of inhibitory activity was lost following 2 and 18 h of incubation of serum at pH 3.7 and was complete after 60 min (Fig. 7). The bands of endogenous glycoproteins, representing false positives, and some of the bands of minor inhibition activity were observed faintly in Fig. 5. These subtle bands were difficult to identify on a routine basis. Observations of such bands were dependent in part on the level and duration of Alcian blue destaining.

Circulating levels of HA are markedly elevated in patients with shock, septicemia, and major burns (57–61). The half-life of circulating HA is from 2 to 5 min. Rapid increases in circulating HA could be accomplished by sudden inhibition of such constant HA catabolism, an activity that could be attributed to an inhibitor of hyaluronidase activity that has the properties of an acute-phase protein. The animal model for inducing the acute-phase response is the intraperitoneal administration of 40 μl of CCl₄ to an adult mouse (62). Serum or plasma samples that contain acute-phase proteins are then obtained after 6, 24, or 48 h. As shown in Fig. 9A, increased levels of hyaluronidase inhibitor levels were obtained (212 and 290% at 24 and 48 h, respectively) compared with control serum levels. IL-1 is an inducer of the acute-phase response. A 1-μg intraperitoneal injection of IL-1 produced a 202% increase in hyaluronidase inhibitor activity at 24 h, but no further increase at 48 h (Fig. 9B).

The effect of purified preparations of mouse Iα on hyaluronidase inhibition was examined next. Increasing amounts of Iα or the Iα-related 120-kDa complex were added to the testicular hyaluronidase reaction mixture. Iα at 0.3, 0.6, and 1.2 μg/ml inhibited the enzyme by 9.05, 15.63, and 24.06%, respec-

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**TABLE II**

<table>
<thead>
<tr>
<th>Cone of anti-Iα antibody</th>
<th>0.50 mg/ml</th>
<th>1.25 mg/ml</th>
<th>2.50 mg/ml</th>
</tr>
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<tbody>
<tr>
<td><strong>A. Loss of neutral hyaluronidase inhibitory activity</strong></td>
<td></td>
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</tr>
<tr>
<td>Mouse serum</td>
<td>% inhibition</td>
<td>Control</td>
<td>0.50 mg/ml</td>
</tr>
<tr>
<td>% of control</td>
<td>100</td>
<td>ND</td>
<td>85.5 ± 12.0</td>
</tr>
<tr>
<td>Human serum</td>
<td>% inhibition</td>
<td>100</td>
<td>55.4 ± 8.0</td>
</tr>
<tr>
<td>% of control</td>
<td>100</td>
<td>48</td>
<td>31</td>
</tr>
<tr>
<td><strong>B. Serum acid-active hyaluronidase remaining following immunodepletion for hyaluronidase inhibitory activity</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mouse serum</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Units</td>
<td>% of control</td>
<td>100</td>
<td>7.08 ± 1.2</td>
</tr>
<tr>
<td>Human serum</td>
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<td></td>
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</tr>
<tr>
<td>Units</td>
<td>% of control</td>
<td>100</td>
<td>14.1 ± 1.0</td>
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Hyaluronidase inhibition activity was measured at 0.5% (v/v) serum and showed decreased inhibitory activity at the higher levels of inhibitor Iα to inhibit the hyaluronidase reaction (data not shown). The inhibitor mixture alone, however, was able to inhibit the hyaluronidase reaction, indicating that the molecules present in the reaction mixture.

Levels of hyaluronidase inhibitor activity in mouse serum were examined at various time points following the in vivo intraperitoneal injection of CCl4 (A) and IL-1 (B). Hyaluronidase inhibition activity was measured at 0.5% (v/v) serum using 1 rTRU/ml bovine testicular hyaluronidase.

**TABLE III**
*Inhibition of bovine testicular hyaluronidase activity by purified mouse Iα and Pα*

<table>
<thead>
<tr>
<th>Addition of purified mouse Iα and Pα</th>
<th>0.3 µg/ml</th>
<th>0.6 µg/ml</th>
<th>1.2 µg/ml</th>
<th>2.4 µg/ml</th>
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</thead>
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<tr>
<td>Iα</td>
<td>9.05 ± 7.79</td>
<td>15.63 ± 11.3</td>
<td>24.06 ± 9.46</td>
<td>17.22 ± 12.7</td>
</tr>
<tr>
<td>Pα</td>
<td>11.24 ± 9.70</td>
<td>18.64 ± 1.84</td>
<td>20.02 ± 1.97</td>
<td>17.41 ± 4.19</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The earliest evidence for circulating inhibitors of hyaluronidase, presented between 1946 and 1949 (21–23), was followed a decade later by attempts at characterization. It was established that they are largely thermolabile, high molecular mass glycoproteins (29) with a requirement for magnesium ion (30). Despite clinical reports describing increases in inhibitor activity levels in association with various disease states (24–28), no subsequent progress was made in characterizing these inhibitors.

This study was undertaken using some of the newer technologies not available at that time. Preliminary experiments demonstrated that rodent serum contained much higher levels of inhibitor than did human serum. Mouse serum was used therefore in most subsequent studies. Physical and biochemical studies documented that the inhibitor activity was thermolabile and proteinase-sensitive and had a magnesium requirement, confirming earlier studies. The predominant circulating hyaluronidase inhibitor was a glycoprotein of ~120 kDa and appeared to be a member of the complex family of Iα proteins. Additional bands containing inhibitory activity with greater molecular size (190 and 220 kDa) and one minor band of lower molecular size (75 kDa) were observed consistently, but were not characterized further. These bands also disappeared following the chondroitinase digestion steps, as did the predominant hyaluronidase inhibitor band (Fig. 8).

The inhibitory activity had little influence on acid-active hyaluronidases, but did inhibit various neutral-active hyaluronidases. A wide range of activity was observed against neutral-active enzymes. The strongest inhibition was against bovine testicular hyaluronidase; modest levels were against bee and snake venom hyaluronidases; and no inhibitory activity could be demonstrated against *Streptomyces* hyaluronidase.

It might be postulated that the putative inhibitor in serum binds to the HA substrate and that the mechanism of apparent enzyme inhibition is to protect substrate from degradation. If this were the case, the enzyme from *Streptomyces* would have also been inhibited. Substrate protection is therefore an unlikely mechanism for the serum inhibitor. A neutral-active hyaluronidase from the leech, which, unlike the others, is an endo-β-glucuronidase rather than an endo-β-N-acetylglucosaminidase (63), would have been of intrinsic interest, but is no longer commercially available.

A treatment of the inhibition reaction by classical kinetic analysis seems unlikely. The HA substrate is bound to the microtiter plate, and undigested product is what is measured. Classical Lineweaver-Burk plot analysis assumes that both enzyme and substrate are soluble. Additionally, it is difficult to formulate how polymer digestion can be treated quantitatively. The secondary structure and folding of the HA molecule undergo progressive changes as the size decreases (64); the volume of bound water of hydration is a nonlinear function; and each physical stage through which the molecule goes, from 107 to 103 kDa down to the limit tetrasaccharides, may have a different affinity for enzyme. Nonetheless, we demonstrated that heparin is a noncompetitive inhibitor of the hyaluronidase reaction. Attempts are underway to examine the plasma inhibitor in a similar fashion.

The biological function of this major serum inhibitor of hyaluronidase activity is of considerable interest. Expression of the gene for the neutral-active sperm-associated hyaluronidase PH-20 is restricted, with certain exceptions, to mammalian testis as detected by Northern blot analysis. Expression is also detectable in fetal and placental cDNA libraries by polymerase chain reaction, suggesting a possible role for this gene during embryonic development (19). Some RNA can also be detected in kidney and prostate libraries (65), although no protein can be detected. We have confirmed these results. Aberrant expression also occurs in certain malignancies (66, 67). The function of the circulating inhibitor is not known, but the expression of one target for this inhibitor during fetal development and in malignancy suggests interesting possibilities.

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*3 J. Moon, S. Gutierrez, and R. Stern, unpublished data.*

*4 Csoka, A. B., unpublished data.*
This enzyme is thought to digest the HA-rich cumulus mass that surrounds the mammalian ovum and that facilitates sperm penetration in the process of fertilization (68, 69). The narrow window at neutral pH for the activity of the plasma hyaluronidase inhibitor may function as a control of cumulus matrix digestion. Small changes of pH can modulate levels of inhibitor activity, a mechanism for female control of fertilization.

The IoI molecules are one major group of Kunitz-type plasma protease inhibitors with a high molecular mass, ranging from 130 to 240 kDa. The original form reported for IoI consists of one light and two heavy chains. The polypeptides are connected by covalent linkages to a chondroitin sulfate chain (70). Further studies have demonstrated the existence of an enormous molecular divergence in IoI family members. In addition to the many genes coding for heavy chains, divergence in the number and combination of peptides in the molecule makes IoI a complex family of molecules. PoI is another member of the IoI family, an unfortunate term, so named because it precedes α₁-globulin in serum electrophoresis and not because it is a precursor to IoI. It consists of one heavy chain and one light chain.

The 120-kDa band of hyaluronidase inhibitory activity was observed by reverse HA substrate gel zymography. Both biochemical and immunological studies suggested that the inhibitor had the characteristics of an IoI-like molecule. Digestion of the glycosaminoglycan by chondroitinase, as shown in these experiments, eliminated hyaluronidase inhibitory activity. The structure resulting from the linkages to chondroitin sulfate appeared to be necessary for the inhibitory activity. Absolute identification of the elusive circulating hyaluronidase inhibitor has not been achieved and will require further investigation. However, the reported molecular size of PoI (120–130 kDa) makes it a promising candidate.

Recent studies indicate that two members of the IoI family, IoI and PoI, are critical in organizing and stabilizing the expanding extracellular matrix of cumulus-oocyte complexes in culture in vitro (35). It has also been reported that proteins of the IoI family can form coherent interactions with HA within the ovulated cumulus extracellular matrix (35). Our results suggest that IoI could participate in the regulation of the hyaluronidase activity of PH-20 during the penetration of the sperm through the cumulus matrix.

Six hyaluronidases are reported to occur in the human genome (19), with a possible seventh uncharacterized activity (20). Serum and plasma contain a single hyaluronidase enzyme, Hyal-1, with activity only in the acid pH range. There is considerable interest in establishing the mechanism and function of Hyal-1 in the circulation, the first somatic hyaluronidase to be isolated and sequenced (19, 43, 71, 72). The gene for Hyal-1, HYAL1, is the central sequence in the cluster of three on chromosome 3p21 (19). Except for PH-20, none of the hyaluronidase-like sequences have products with activity at neutral pH. Despite intense efforts, the identification of the natural physiological target for this circulating neutral-active inhibitor of hyaluronidase remains unknown.

Some investigators have speculated that the serum hyaluronidase inhibitor is a natural protection system against invasion by microorganisms. Hyaluronidase, the identity of the original "spreading factor," does facilitate penetration of venoms and microorganisms. However, as described in these studies, the circulating hyaluronidase inhibitor has no ability to inhibit Streptomyces hyaluronidase. Therefore, it cannot be invoked as a natural defense mechanism for microbial invasion into tissues. An inhibitor in saliva active against microbial hyaluronidases that is also active at neutral pH was recently identified in this laboratory (73). However, it could not be ascertained with certainty whether the inhibitor was of host or of microbial origin.

There have been investigations attempting to suppress experimental venom penetration in vivo by injecting types of hyaluronidase inhibitors in advance. No success has been achieved using heparin or mammalian serum. However, some flavonoids and tannic acid are reported to prolong survival in mice following subcutaneous injection of venom (74). Flavonoids (75–77) and tannins (78) are plant-derived hyaluronidase inhibitors, as are hydrangenols from hydrangea (79); curcumin from the spice curcumin (80); glycyrrhizin from licorice (81); and tranilast, a chemically modified cinnamic acid (82). Some of these compounds (77) and their synthetic derivatives (83) have also been shown to have modest abilities as contraceptives, presumably through their ability to inhibit sperm hyaluronidase activity.

Certain anti-inflammatory drugs have also been reported to possess hyaluronidase inhibitor activity. These include salicylates (84) and indomethacin (85). Some of the same plant extracts mentioned above are used in folk medicines as anti-inflammatories (86).

These compounds have been used traditionally in folk remedies and are described in the growing area of ethnopharmacology. All these plant-derived inhibitors are far less potent against neutral-active hyaluronidases than the circulating inhibitor described here. Hyaluronidase inhibitors of animal origin show promise for intervention in a number of human disorders.

In summary, a new class of biologicals is described, hyaluronidase inhibitors, whose activities were first detected half a century ago (21–23). Some of these inhibitors may belong to the IoI family of molecules. It appear likely that not all hyaluronidase inhibitors belong to the IoI family. Only circulating inhibitors were examined here. Tissue-associated hyaluronidase inhibitors may exist, and there may be others in the circulation that were not detected in these studies. These may not have been detected in the artificial in vitro assay system utilized here, or they may have been irreversibly denatured following exposure to SDS. The hyaluronidase inhibitors may turn out to be a family of molecules more complex than the hyaluronidases themselves (9).

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Evidence That the Serum Inhibitor of Hyaluronidase May Be a Member of the Inter-α-inhibitor Family
Kazuhiro Mio, Odile Carrette, Howard I. Maibach and Robert Stern

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