

Heparin-like anticoagulants in asthma

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In a previous study, and in the present study, we have found that the baseline plasma samples of patients with asthma contain average levels of an endogenous heparin-like material (EHM) that is significantly higher than that noted in non-allergic, non-asthmatic controls. This material appears to have properties of both heparin and heparan sulfate. Three out of six patients responding to inhalational antigen challenge displayed an acute increment in EHM concentration that coincided with a fall in FEV₁ values. The relation of EHM concentration to provoked asthma, or to asthma in general, remains to be determined.

Key words: asthma; heparin; heparan sulfate; heparinemia.

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Circulating heparin-like anticoagulants have only rarely been described in humans (12, 22, 24, 26, 33). These have occurred in hematologic neoplasia (12, 24, 33), systemic mastocytosis (22), carcinoma of the esophagus with bone marrow invasion (33), and inflammatory bowel disease with hepatitis (26). We have previously reported a mean elevated level of heparin-like anticoagulants in baseline plasmas of a group of atopic asthmatics (15). Utilizing techniques to minimize platelet aggregation in sample processing and to displace the anticoagulant from protein neutralizers, we now report that 22 asthmatics demonstrated plasma levels of an anti-Xa substance that averaged 0.076 heparin equivalent units/ml, whereas controls had essentially none, and that an elevation of this endogenous heparin-like material (EHM) can be induced in some patients after antigen inhalational challenge.

MATERIAL AND METHODS

Twenty-two patients (mean age 41; 13 F; 9 M) undergoing diagnosis and therapy in the Clinical

Allergy and Immunology Division of Scripps Clinic and Research Foundation volunteered for this study with informed consent, and with the approval of the Human Subjects committee. The asthmatic patients included in this report all had documented reversible airways obstruction. Three had non-specific bronchial hyperactivity (skin-test and aspirin or metabisulfite challenge negative), eight were skin-test positive to one or more common allergens, and the remainder reacted positively to either aspirin or metabisulfite oral challenge. Seven patients with atopic asthma agreed with informed consent to undergo bronchial inhalational challenges with antigens to which they had a documented skin hypersensitivity. Standard challenge techniques were utilized (29). Antigens, in 1 ml solution at varying concentrations, were nebulized by a French-Rosenthal dosimeter (Laboratory for Applied Immunology, Inc., Johns Hopkins). Control blood samples were obtained from individuals coming to excretory urography in either the University Hospital of the University of California, San Diego, School of Medicine, or the associated Veterans Admin-

istration Medical Center. These were obtained with informed consent from patients with a clearly negative history of allergy or asthma and were processed in exactly the same way as patient samples. These patients were not subjected to inhalational challenge and were not skin-tested. Blood samples were drawn into plastic vials containing EDTA, 2-chloro-adenosine and procaine-HCL (Thrombotect tubes, Abbott Laboratories, Chicago). In one inhalational challenge, patient blood was collected in citrated plastic vials. Arterial and venous samples, or arterial samples alone, were drawn prior to challenge, and at intervals up to 60 min post-reaction. Citrated samples were held at room temperature and Thrombotect samples were held on ice for 1 h prior to processing. They were then transferred to polypropylene tubes and centrifuged at 2,000 rpm for 10 min. After transfer of the supernatant to a second polypropylene tube, additional centrifugation was carried out at 10,000 rpm for 10 min to produce platelet-free plasma. Plasmas were stored at -70°C prior to assay. Anti-Xa EHM assays were carried out by a slight modification of the method of Teien & Lie (31). In this modification, a single normal human plasma (NHP), known to be completely free of EHM activity, was used as a source of antithrombin III (AT-III), as a vehicle for dilution, when necessary, and as a plasma for constructing a standard anti-Xa heparin curve. All determinations were done in duplicate. In some plasmas, the effect of added protamine sulfate (Eli Lilly Co., Indianapolis, IN) on anti-Xa EHM was determined. The effects on EHM of heparinase and chondroitinase ABC (Miles Scientific, Naperville, IL) were also tested. The heparinase had been prepared from *Flavobacterium heparinum*. The chondroitinase from *P. vulgaris*.

Anticoagulant EHM assays can account for only that percentage of EHM that is not bound to neutralizing proteins, such as platelet factor 4 (PF4), β -thromboglobulin and others. Under these circumstances, the amount of anticoagulant EHM mobilized into the circulation in a given situation is likely to be underestimated. The extent to which this occurs will depend

upon the level of circulating neutralizing proteins at the time of sampling. It is known that antigen challenge is commonly followed by an increase in the level of circulating PF4 (14), possibly due to the prior cellular release of platelet-activating factor. Therefore, a method was devised to totally liberate a given concentration of added commercial heparin in the presence of binding protein. This was accomplished by the addition of a substance-low molecular weight (8,000 daltons) dextran sulfate (Sigma Chemical Co., St. Louis, MO) in a concentration that would competitively displace heparin from its plasma binding sites without interfering with its binding to AT-III. In preliminary studies, it was determined that, for plasma samples containing ADP-induced platelet release products induced by incubation of whole blood with 2×10^{-4} molar ADP, or products derived from sequential platelet freeze-thaw sequences, a final dextran sulfate concentration of 0.12 mg/ml of plasma would assure the total release of added anticoagulant heparin (porcine heparin - Riker Laboratories, Northridge, CA) in the range of 0.01 to 0.8 U/ml. For higher concentrations of heparin, plasma dilutions, maintaining the original dextran sulfate concentration, can be employed. Since EHM has the anti-Xa (and antithrombin) properties of heparin, and like heparin, is neutralized by the addition of protamine sulfate or PF4, it was presumed that the addition of dextran sulfate would correspondingly liberate anti-Xa EHM. This was confirmed by adding (1:2) a plasma containing 0.23 U/ml of EHM to the plasma containing platelet release products and determining that the entire anti-Xa EHM concentration was recoverable after the addition of dextran sulfate. Dextran sulfate, in the presence of AT-III at the concentrations used for the anti-Xa assays, had little or no anti-Xa properties of its own. Standard reference curves, constructed with either purified AT-III (Helena Laboratories, Beaumont, TX) or a standard laboratory plasma, were identical when the dextran sulfate displacement technique was utilized to construct the plasma curve. Therefore, assays done with and without the addition of dextran sulfate were referred to

Table 1

When plasma samples were assayed with dextran sulfate (DS) to uncover cryptic EHM, there was a significant difference in the found concentrations in asthmatics in comparison with controls

	EHM (heparin-equivalent U/ml)		<i>P</i>
	Asthma (22)	Controls (29)	
Assay with H ₂ O	0.055 ± 0.035 ¹	0.001 ± 0.000	>0.05 < 0.1
Assay with DS	0.076 ± 0.045	0.001 ± 0.001	0.05

¹SEM.

the standard plasma curve done with dextran sulfate to avoid ambiguities that might otherwise occur because of EHM-neutralizing proteins. The utilization of AT-III reference curves (or, in this case, an equivalent AT-III technique) rather than plasma reference curves has been shown to increase the sensitivity of the anti-Xa heparin assay (31).

RESULTS

For concentrations of EHM in the range of 0.0 to 0.049, the mean difference between 31 duplicate aliquot samples assayed on different days (± SD) was 0.005 ± 0.006 U/ml. For concentrations of EHM in the range of 0.05 to 0.5 U/ml, the mean difference between 29 duplicate samples done on separate days was 0.03 ± 0.03 U/ml. The correlation coefficient for 59 duplicate EHM determinations done on separate days (range 0.0 to 0.5 U/ml) was 0.9762.

Twenty-two patients with asthma and 29 controls had early morning pre-challenge plasma samples assayed for EHM with and without the utilization of the dextran sulfate displacement techniques. Without dextran sulfate, the

asthma plasmas averaged 0.055 U/ml of EHM vs. 0.000 U/ml in controls. With dextran sulfate the asthmatics averaged 0.076 U/ml, and the controls 0.001 U/ml, a significant difference at *P* = 0.05 (Tables 1 and 2). Twelve of the 22 patients were receiving systemic corticosteroids at the time the blood samples were obtained. Almost all patients had sufficiently severe disease that they were on one or more drugs for asthma or allergic rhinitis. However, there was no obvious correlation between drug history, perceived severity of the disease, state of the disease at the time blood samples were drawn, and EHM levels. In further studies (unpublished data), it was noted that the presence or absence, and/or the concentration of venous EHM may vary in a given asthmatic patient on a day-to-day basis. In individuals with a definite negative history of allergy, however, increased EHM concentrations have not yet been detected. These findings suggest that a mobilizing factor, not yet identified, may be present intermittently in asthmatic patients.

Incubation of a patient plasma containing an EHM concentration of 0.47 U/ml with 1 U/ml of heparinase at 37°C for 4 h, produced a 25 % loss of anti-Xa EHM. Exogenous heparin added to NHP at the same final concentration and incubated for 4 h at 37±C, showed a 31 % loss of anti-Xa EHM. In another patient plasma containing 0.43 U/ml of EHM, 10 U/ml of heparinase produced a 70 % loss of anti-Xa EHM over 4 h at 37°C. With the same heparinase concentration, there was a loss of 73 % of equivalent exogenous heparin in NHP over the same period. Controls incubated without heparinase, or with heat-inactivated heparinase, showed no change. When 10 U/ml of

Table 2

The majority of control plasmas have < 0.005 EHM (U/ml), whereas the majority of asthmatic plasmas contained between 0.006 and 0.5 EHM (U/ml)

EHM (U/ml)	Assay with DS (range values)	
	Asthma (<i>n</i>)	Control (<i>n</i>)
0.000–0.005	7	26
0.006–0.050	9	2
0.060–0.500	5	0
0.600–1.000	1	0

heparinase and 0.43 U/ml of EHM from the same patient were incubated for 4 h at 43°C, a temperature optimal for the hydrolysis of heparan sulfate by heparitinase, but not for the hydrolysis of heparin by heparinase (19), the loss of anti-Xa heparin did not exceed that noted in a control incubation without heparinase. Thus, the enzyme-mediated loss of EHM at 37°C was not due to contaminating heparitinase, but due to the heparin specific heparinase (2). Four-hour, 37°C incubations of 1 U/ml and 10 U/ml of chondroitinase ABC with a plasma containing 0.42 U/ml of EHM failed to produce any loss of anti-Xa EHM. Isolation of EHM from pooled platelet-free patient plasmas was accomplished by a modification of the method described by Palmer et al. (24) in which DEAE Sephacel column anion exchange chromatography and elution with 3.0 M KCl was followed by dialysis. 10 ml DEAE-cellulose (DE53, Whatman) columns previously equilibrated with 0.01 M sodium acetate and 0.1 M sodium chloride were utilized. Elution produced a partially purified product that lost 100 % of anti-Xa activity on 4 h, 37°C incubation with heparinase. No anti-Xa activity was lost on incubation of this sample with chondroitinase ABC for 4 h at 37°C. Subsequent electrophoresis of the partially purified product in lithium acetate buffer (8) after pronase digestion, and comparison to N.I.H. glycosaminoglycan standards (kindly supplied by Dr. A. Horwitz, Department of Pediatrics, University of Chicago) showed that EHM migrated, in part, with heparan sulfate, and, in part, to a region intermediate to chondroitin-6-sulfate and hyaluronic acid. No co-migration with heparin was demonstrated. Thus, it appears that EHM resembles the heparin-like material isolated from cerebral microvessels by Marcum & Rosenberg (20, 21). This material has considerably more anticoagulant activity than heparin-like species derived from whole aortas, but has the structural characteristics of heparan sulfate rather than heparin fragments (21). When protamine sulfate (final concentration 70 µg/ml) was added to asthma plasmas containing EHM, the anti-Xa heparin activity disappeared.

Seven patients with atopic asthma were chal-

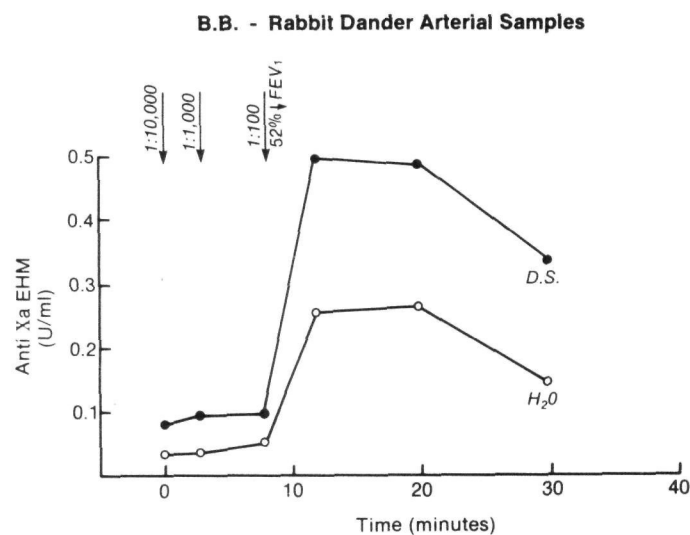


Fig. 1. Concentrations of anti-Xa EHM at varying time periods in the plasma samples of a patient responding to inhalational antigen challenge. The patient experienced a 52 % fall in FEV₁ when challenged with a 1:100 dilution of rabbit dander. Assays were done with 8,000 MW dextran sulfate to expose cryptic EHM (D.S.) and without dextran sulfate (H₂O). The difference in concentrations at any time period thus represents a measure of cryptic EHM at that period.

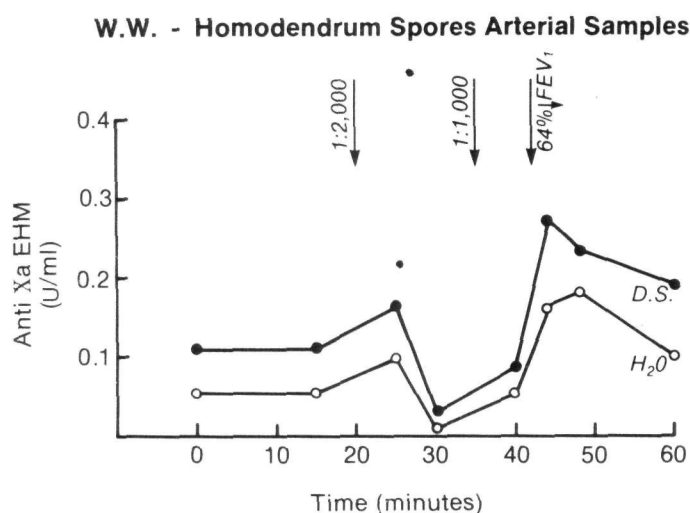


Fig. 2. See legend for Fig. 1.

lenged with antigens to which they had demonstrated a positive skin reaction. Three of the seven developed increased EHM arterial plasma concentrations in response to the challenges. Concurrently, or shortly thereafter, these patients demonstrated a fall in FEV₁ values of 64 %, 52 %, and 19 % (Figs. 1, 2). Two patients showed only a slight increase in EHM, but responded to the challenge with a fall in FEV₁

values of 29% and 17%. One patient responded with a fall in FEV₁ of 36%, but displayed no EHM and one patient did not significantly deviate from baseline FEV₁ values and did not increase his baseline EHM concentration. This patient showed only an 11% fall in FEV₁, following a 1 ml inhalational challenge with a 1:100 dilution of mixed grasses. Peak arterial EHM concentrations in these seven patients correlated positively with FEV₁ decrements ($r = 0.7899$; $P < 0.05$) (Table 3).

DISCUSSION

Asthmatic individuals have elevated mean levels of EHM. Inhalational antigen challenge in sensitized individuals may result in a fall in FEV₁ values and the arterial plasma mobilization of EHM. This endogenous heparin-like material is partially neutralized by binding substances in plasma, but can be displaced from these binding sites by appropriate concentrations of low molecular weight dextran sulfate. The nature of the binding substances was not ascertained, but the antigen-induced elevations and the heparin-neutralizing potential that have previously been determined for PF4 and β -thromboglobulin (14, 17, 27) would not appear to be sufficient to account for the heparin (or EHM) binding capacity noted in Fig. 1 and, possibly in Fig. 2, following the induced reaction. Furthermore, ADP-aggregated plasma with a PF4 concentration approximately 30 $\times$ that found at the zenith of EHM in Case 2 could not neutralize more than 0.1 U/ml of heparin or EHM (data not shown). Other substances, however, such as

eosinophilic cationic protein (12), might play a role.

In a previous study, we reported that the average concentration of endogenous heparin-like material active with AT-III against thrombin was significantly elevated in the plasma of a group of atopic asthmatics undergoing routine outpatient therapy for their asthma (15). In that study, the concentration of endogenous heparin-like material active against Xa did not quite attain statistical significance, but at that time we were not using a technique to unmask neutralized heparin.

The EHM that we are dealing with has functional properties that are similar to those found with commercial heparin. Specifically, it shows an accelerated inhibition of Factor Xa or thrombin in the presence of AT-III, can be neutralized by protamine sulfate, and acts as an immediate anticoagulant when mixed with normal plasma. Furthermore, like commercial heparin, it is hydrolyzed by heparinase, but not by chondroitinase ABC. Thus, the material has, at least, some of the characteristics of commercial heparin. Its electrophoretic comigration with heparan sulfate indicates that it must have some of the structural properties of this substance as well. The presence or absence of a protein constituent and other molecular characteristics will be further ascertained when sufficient material has been isolated for these analyses.

The source of EHM in these patients and in those described in the few other reports appearing in the literature (12, 22, 24, 26, 33) is not known. In animal studies, immunologically

Table 3

Peak arterial EHM concentrations and maximal drop in FEV₁ in seven patients challenged with inhalational antigens to which they have a positive skin reaction. Peak EHM concentrations and drop in FEV₁ correlated at $r = 0.7899$ ($P < 0.05$)

Patient	Antigen	↓FEV ₁ (%)	Peak arterial EHM (U/ml)
1	homodendrum spores	64	0.29
2	rabbit dander	52	0.46
3	mite	36	0.00
4	mixed grasses	29	0.02
5	cat dander	19	0.05
6	mixed grasses	17	0.03
7	mixed grasses	11	0.01

induced mast cell activation resulted in degranulation with a linear release of preformed histamine and heparin contents, but heparin, unlike histamine, remained in close association with the cell following discharge (34). The presence of a heparin-like material has been noted in the plasmas of dogs and guinea pigs following antigen-induced shock (6, 10), but, to our knowledge, the presence of a similar substance following antigen challenge in humans has not previously been reported.

A heparin-like anticoagulant has been found to be present in endothelial cell cultures (1). An endoglycosidase, present within platelet lysosomes (2, 9, 23), has been noted to both liberate and degrade heparan sulfate, a proteoglycan possessing heparin-like anticoagulant properties present on the surface of endothelial cells (2). Platelets themselves have been reported to release heparin after *in vitro* adhesion has been induced (32).

In earlier studies, we noted that the plasmas of atopic asthmatic patients (15) and X-ray contrast material reactors (16) showed a more rapid transformation of prekallikrein to kallikrein on incubation with a soluble contact activator than did matched controls, and this was potentiated by the addition of heparin (15). An increased rate of contact system activity has also been noted by others in animals or humans displaying anaphylactic responses to antigen challenge (4, 25). In the contact system cascade, the production of kallikrein precedes the release of bradykinin, a direct and indirect putative mediator in experimental and human anaphylaxis (3). Contact activators are known to be present in endothelial cells, and heparin itself will activate the contact system (3, 7). Therefore, the plasma mobilization of EHM might be considered as a possible initiator, and/or potentiator of contact system activity, possibly preceding anaphylaxis. Other studies also suggest a possible role for EHM in anaphylaxis. For example, heparin, when mixed with trichinella antigens, produced cutaneous anaphylactic responses in mice that were equivalent to those produced with Freund's complete adjuvant (11). Heparin also enhanced or potentiated IgE and IgG anti-mellitin antibody formation in

mice (13), and increased the release of histamine from human leukocyte passively sensitized with sera from ragweed-sensitive donors via an enhancing effect on antibody-cell complexing (18). In other studies (30), heparin functioned as a T-cell mitogen (human) and polyclonal B-cell activator (mouse).

The presence of EHM in allergic, non-asthmatic individuals has to be further determined as does the actual role of EHM, if any, in asthma and anaphylaxis. For the present, it is important to note that elevated concentrations of this substance are present in the plasmas of some patients suffering from asthma and certain drug sensitivities, and that, in some instances, these concentrations may be further elevated following antigen-induced bronchospasm or drug-induced anaphylactoid reactions.

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