Effect of Trypsin Inhibitors on Shwartzman Phenomenon.* (27004)

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Despite the similarity of some of the pathologic findings in varied abnormal states such as the Shwartzman phenomenon, the Arthus phenomenon and other antigen-antibody reactions, and certain types of allergy and shock, neither a common factor nor a fundamental mechanism responsible for the pathogenesis of these reactions has been established. Some of these reactions may be induced by bacterial endotoxins which, in all likelihood, act indirectly via one or more intermediate steps. There is increasing evidence that the mechanism of these phenomena involves a complex chain of reactions in which enzyme activities may play important roles. Intracellular proteolytic enzymes have been considered essential factors in production of the Shwartzman phenomenon(1,2) and trypsin was reported to potentiate this phenomenon(3). In addition the blood coagulation process, an important element in the Shwartzman reaction(4,5), is also influenced by proteolytic enzymes. Besides, bacterial endotoxins induce fibrinolytic activity *in vivo*(6,7,8), and are also thought to activate other proteolytic enzymes which are distinct from the plasminogen-plasmin system (9). Participation of such enzymes have been suggested in the mechanism of endotoxin induced shock(9). Recently, *in vitro* potentiation of serum proteolytic activity by endotoxins was also reported(10). These proteolytic enzymes might activate plasma kininogens which in turn effect tonicity and permeability of blood vessels. In view of these considerations a study of the effect of trypsin inhibitors on the Shwartzman phenomenon was undertaken (11).

Materials: 160 albino female rabbits, weighing between 1.7 and 2 kg, were used in these experiments. They received Rockland rabbit ration, (antibiotic-free) and water *ad libitum* and were housed in air-conditioned animal rooms at about 65°F. The abdominal skin was depilated with an electric clipper.

The inhibitors employed were crystalline pancreatic trypsin inhibitor, crystalline soybean trypsin inhibitor, and crude soybean trypsin inhibitor obtained from Mann Research Laboratories.

Solutions of *E. coli* endotoxin (Difco) in concentrations from 3 μg/ml to 500 μg/ml were used for skin preparation and 250 μg/ml for all provocative intravenous injections. A volume of 1 ml was used for each intradermal or intravenous injection. All solutions in these experiments were made in pyrogen-free normal saline.

Method: Exp. I. In 34 rabbits varying amounts of endotoxin ranging from 6 to 500 μg were injected intradermally at different sites along one flank of each animal. The same amounts of endotoxin mixed with one of the trypsin inhibitors were injected at corresponding sites of the other flank. The amount of trypsin inhibitor in each injection varied from 6 μg to 10 mg. Crystalline pancreatic trypsin inhibitor was used for 6 rabbits (20 sites), crystalline soybean trypsin inhibitor for 3 rabbits (9 sites) and crude soybean trypsin inhibitor for the remaining animals. The provocative endotoxin injection was intravenously administered 24 hours later.

Exp. II. 6 rabbits in a control group and 10 rabbits in a second group were injected intradermally at various sites of the abdominal skin with 6, 12, 25 and 50 μg of endotoxin. Within a period of 5 minutes before or after these intradermal injections the second group received 500 μg of a trypsin inhibitor in 1 ml of saline intravenously. Crude soybean trypsin inhibitor was used for 2 rabbits and crystalline soybean trypsin inhibitor for the remaining 8 animals. The provocative intravenous injection of endotoxin was administered 24 hours later to both groups.

Exp. III. Various amounts of endotoxin ranging from 12 to 100 μg were intradermally injected at various sites along one flank of 47 rabbits. The same amounts were injected at

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corresponding sites of the other flank. Each injection site was carefully marked with a circle. Twenty-four hours later 0.5 ml of saline was injected in all sites of one flank which served as controls. The same volume of saline containing 50 μg to 3 mg of one of the trypsin inhibitors was injected in the corresponding sites of the other flank. Crystalline soybean trypsin inhibitor was injected in 3 rabbits (7 sites), crystalline pancreatic trypsin inhibitor in 2 rabbits (2 sites) and crude soybean trypsin inhibitor in the remaining animals. The provocative injection of endotoxin was administered within a period of 30 minutes before or after the intradermal inhibitor injection.

Exp. IV. Twenty-eight rabbits in the control group and 37 in a second group were injected intradermally at different sites of the abdominal skin with various amounts of endotoxin ranging from 3 to 100 μg. The provocative intravenous injection of endotoxin was administered 24 hours later to both groups. The second group received in addition 500 μg of one of the trypsin inhibitors in 1 ml of saline intravenously simultaneously with or at intervals ranging from 5 to 120 minutes before or after the provocative endotoxin injection. Of the 37 rabbits in this group 4 were treated with crystalline soybean trypsin inhibitor, 3 with crystalline pancreatic trypsin inhibitor and 30 with crude soybean trypsin inhibitor.

Results: The skin reactions in all experiments were read 5 hours after the provocative endotoxin injection.

Exp. I. Trypsin inhibitors administered intradermally mixed with the endotoxin skin preparatory dose. In the majority of the animals (74%) the intradermal injection of trypsin inhibitors in combination with endotoxin did not influence the skin preparatory ability of the latter. The number and intensity of positive reactions for each preparatory dose of endotoxin was about the same in both control and inhibitor injected sites (Fig. 1). (In 19% of the animals the reaction in the sites injected with trypsin inhibitors was slightly weaker and in 7% slightly stronger than in the corresponding control sites. These variations in the intensity of reaction are statistically insignificant.) No differences were noted in respect to type or amount of trypsin inhibitor employed. Incubation of the endotoxin-trypsin inhibitor

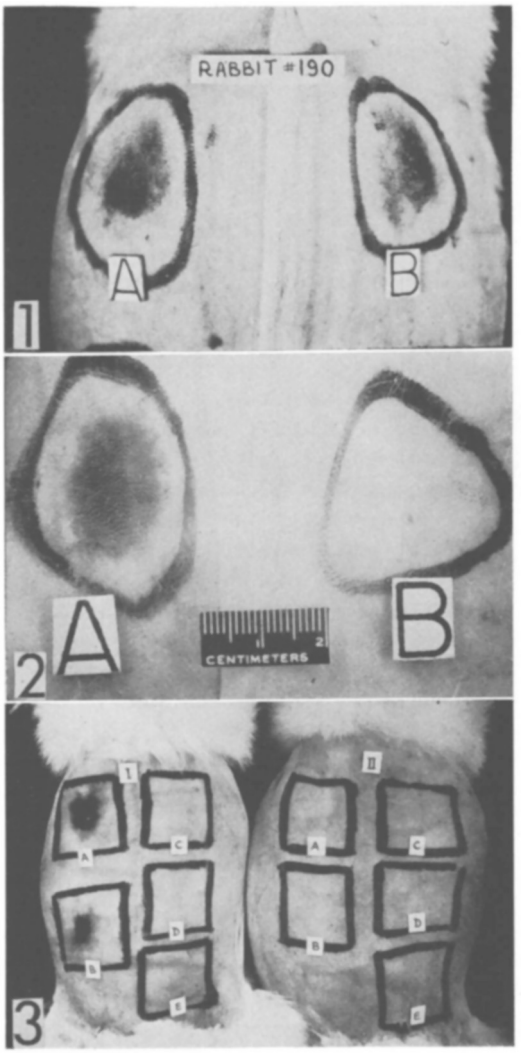

FIG. 1. Site A intradermally injected with 50 μg endotoxin alone. Site B intradermally injected with 50 μg endotoxin mixed with 50 μg trypsin inhibitor. Equal Shwartzman reactions after provocative endotoxin dose.

FIG. 2. Sites A and B intradermally injected with 50 μg endotoxin. 24 hours later (30 minutes prior to provocative endotoxin injection) site A intradermally injected with saline (1 ml) and site B with trypsin inhibitor (2 mg in 1 ml saline). Reaction in site B inhibited.

FIG. 3. Sites A, B, C, D and E in both rabbits intradermally injected with 50, 25, 12, 6 and 3 μg endotoxin respectively. Control rabbit I (left) after the provocative endotoxin dose produced positive reactions at sites A and B. In rabbit II (right) which received 500 μg trypsin inhibitor intravenously 15 min. prior to the provocative endotoxin injection, reactions were inhibited in all sites.

TABLE I. Effect of trypsin inhibitors on the local Shwartzman phenomenon.

Endotoxin intradermal dose (μg)	% positive skin reactions		% strong positive skin reactions*	
	Control rabbits	Trypsin inhibitor treated rabbits	Control rabbits	Trypsin inhibitor treated rabbits
100	100 (5/5)**	100 (8/8)	60.0 (3/5)***	50.0 (4/8)
50	96.6 (28/29)	75.0 (27/36)	36.6 (17/29)	8.3 (3/36)
25	89.3 (25/28)	56.7 (21/37)	50.0 (14/28)	10.8 (4/37)
12	75.0 (21/28)	29.7 (11/37)	14.3 (4/28)	0 (0/37)
6	47.8 (11/23)	17.2 (5/29)	0 (0/23)	0 (0/29)
3	30.4 (7/23)	3.7 (1/27)	0 (0/23)	0 (0/27)

* 3+ or 4+ reaction. (Positive reactions were graded from 1+ to 4+ according to intensity and size of the hemorrhagic area.)

** Number of positive reactions/number of animals.

*** Number of strong positive reactions/number of animals.

mixture for 30 minutes at 37°C before administration to 6 of the animals of this experiment did not alter the results.

Exp. II. Trypsin inhibitors administered intravenously at about the time of skin preparation. Trypsin inhibitors injected intravenously at intervals ranging from 1 to 5 minutes before or after skin preparation did not inhibit production of the local Shwartzman phenomenon. In a few cases there were differences in the intensity of the reaction between control and trypsin inhibitor treated groups, but these differences did not exceed normal variations in reactivity of individual animals. No differences were noted in respect to the inhibitor employed or to the time relationship between skin preparation and inhibitor administration.

Exp. III. Trypsin inhibitors injected intradermally 24 hours after skin preparation into the primed skin sites. In many animals trypsin inhibitors abolished the reaction completely (in 39 of 99 injection sites) (Fig. 2), or limited the skin hemorrhage to a zone surrounding the site of inhibitor injection. In the corresponding control sites which were injected with normal saline, the reaction was never completely inhibited. In some cases, however, there was a small area around the puncture mark of the saline injection which remained pale.

In this experiment although trypsin inhibitors influenced the reaction in a considerable number of animals, there was no apparent correlation between degree of inhibition and amount of inhibitor used or dose of preparatory endotoxin injection. The time relationship between inhibitor injection and administration of the provocative endotoxin dose did not influence the results.

Exp. IV. Trypsin inhibitors administered intravenously at about the time of provocative endotoxin injection. In the group treated with trypsin inhibitors the reactions were considerably inhibited (Fig. 3). Table I summarizes results in this experiment. All concentrations of endotoxin except the 100 μg one, produced fewer positive reactions in the inhibitor treated group than in the control animals. As concentration of endotoxin decreases the effect of the trypsin inhibitor is more striking. In the inhibitor treated group, not only was the number of positive reactions considerably smaller than in the control group, but also the intensity of positive reactions was less pronounced (Table I). Statistical evaluation of the results obtained in this experiment indicates that the differences in reactions between controls and inhibitor treated animals are significant at high levels of confidence. ($P = 0.001$-0.01 for differences in frequency of reaction; $P = 0.03$-0.04 for differences in intensity of reaction). The effect of trypsin inhibitors was most pronounced when administered before the provocative endotoxin injection, less pronounced when administered simultaneously and least evident when administered after the provocative endotoxin injection. No differences were noted in respect to the inhibitor employed.

In addition to the inhibition of the local Shwartzman reaction trypsin inhibitors also prevented renal changes seen in the generalized Shwartzman phenomenon. These changes which were present in 10 out of 28 control rabbits were found in only 1 of 37 inhibitor treated rabbits.

Discussion: The experiments herein reported indicate that trypsin inhibitors administered intravenously at about the time of the provoca-

tive endotoxin injection inhibit the local Shwartzman phenomenon. It is not unreasonable to assume that trypsin inhibitors influence the Shwartzman phenomenon by interfering with proteolytic enzymes which apparently play a role in the mechanism of this phenomenon. Injection of trypsin inhibitors directly into the primed skin sites 24 hours after skin preparation also influenced the local Shwartzman reaction. To a limited extent this effect might be attributed to physical factors related to the volume of the injected fluid since saline injections directly into control sites also, in a few instances, inhibited hemorrhage in a focus about the injection point.

The inhibitors mixed with the intradermal endotoxin preparatory dose, or given intravenously at time of skin preparation, failed to inhibit the Shwartzman phenomenon. This lack of effect at the time of skin preparation may be due to the fact that one or several enzymes which are inhibitable by trypsin inhibitors are not present at this time, being activated or released only after a lapse of time following skin preparation or after the provocative endotoxin injection. A similar delay in activation has also been observed with the permeability factor in diluted plasma(12). Other possibilities to be considered are that proteolytic enzymes are present at the time of skin preparation but their inhibition at this phase of the phenomenon is not critical for development of the reaction or an excess of autochthonous inhibitor is normally present at time of skin preparation and injection of additional inhibitor has no effect.

We observed visceral lesions, after eliciting the local Shwartzman reaction which were indistinguishable from those seen in the generalized Shwartzman phenomenon; this in all likelihood was due to escape of endotoxin from the local skin sites into the lymphatics and in turn into the blood stream. Trypsin inhibitors intravenously administered at time of the provocative endotoxin injection prevented development of these lesions. It may be postulated that proteolytic enzymes are involved in both the local and generalized Shwartzman reaction. The fact that trypsin inhibitors influenced the Shwartzman reaction while epsilon aminocaproic acid (EACA), which primarily prevents conversion of zymogens into the active enzymes failed to do so(13), favors the hypothesis that enzymes involved in the Shwartzman phenomenon are released in their active form from cells or from enzyme-inhibitor complexes. This does not completely exclude zymogen activation which is not affected by EACA. The enzymes involved in the Shwartzman phenomenon may produce tissue changes directly or through release of histamine, heparin, serotonin and/or activation of plasma kinins(14, 15). These factors which have vasomotor effects and can alter the blood coagulation process and vascular permeability may be essential for this phenomenon and other tissue reactions mentioned previously.

Summary. 1 Trypsin inhibitors injected intravenously at about the time of the provocative endotoxin injection, partially or completely inhibited the local Shwartzman phenomenon and prevented development of renal lesions (as seen in the generalized Shwartzman phenomenon). 2 Inhibitors administered intradermally mixed with the endotoxin skin preparatory dose, or intravenously at time of skin preparation, did not influence the reaction. 3 The data presented suggest that activated proteolytic enzymes are involved in the Shwartzman phenomenon. 4 The role of these enzymes in the pathogenesis of the phenomenon is discussed. Alterations of the clotting mechanism and vascular changes brought about by release of serotonin, histamine and activation of plasma kinins have been considered.

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Hyaluronidase Inhibitor and Trypsin Inhibitor in Preparations from Human Urine.* (27005)

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Shulman(1) and Astrup et al.(2) have elaborated methods for isolating the trypsin inhibitor in human urine, and preparations with high inhibitor content have been obtained. These preparations also inhibit chymotrypsin and plasmin.

Recently it was observed that the hyaluronidase in human urine is strongly inhibited at pH 4.63 by a substance in the urine(3). The present communication shows that hyaluronidase is also inhibited by preparations purified by Astrup et al., and that concentrations of trypsin inhibitor and hyaluronidase inhibitor in preparations of different degrees of purification, parallel each other.

Methods. Trypsin inhibitor concentrations were estimated on casein by the biuret method (4). One trypsin inhibitor unit is the amount which inhibits 1 μg crystalline trypsin, containing 24.4 Anson units per gram (Novo Laboratories, Copenhagen).

Five preparations containing varying concentrations of trypsin inhibitor were prepared from human pregnancy urine and placed at our disposal by Dr. Tage Astrup. Prep. I contained 56.4, Prep. II 187.5, Prep. III 234, Prep. IV 333 and Prep. V 612 trypsin inhibitor units per mg. Preparations I to IV were prepared as before(2). Preparation V had been further purified (Astrup, to be published).

Hyaluronidase activity was estimated viscosimetrically(3), using USP Reference Standard hyaluronidase (bovine testis). Thirty μg of hyaluronidase preparation and different amounts of Prep. V (Fig. 1) or of preparation I to IV (Table 1), dissolved in 30 μl acetate buffer (pH 4.63) containing albumin, were added to 150 μl of the substrate (details in (3)).

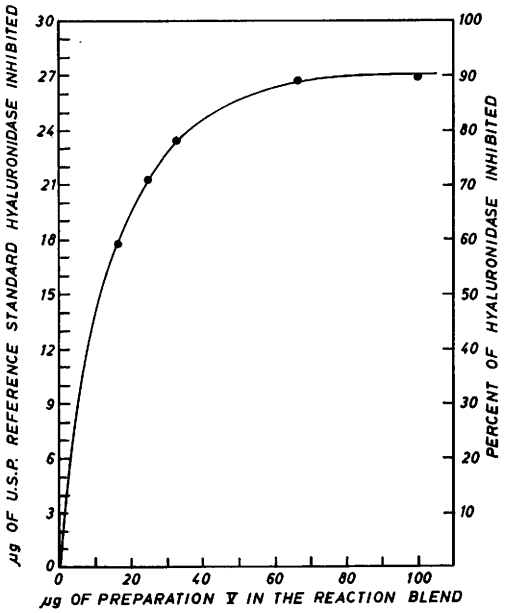

FIG. 1. Inhibition of USP Reference Standard Hyaluronidase by different amounts of a purified urinary trypsin inhibitor preparation containing 612 trypsin inhibitor units per mg (Preparation V). *Abscissa,* μg of trypsin inhibitor preparation in the reaction blend. *Ordinate,* Inhibited amount of hyaluronidase expressed in % of total amount present and μg of hyaluronidase inhibited.

Results. Fig. 1 shows that preparation V, the purest of the trypsin inhibitor preparations, inhibits testis hyaluronidase. The curve indicates a dissociable binding between hyaluronidase and hyaluronidase inhibitor. It is inter-

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