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Inhibition of the Local Hemorrhagic Shwartzman Reaction by a Polypeptide Possessing Potent Antiprotease Activity. (28889)

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The mechanism by which the local "preparatory" injection predisposes the tissue to the hemorrhagic Shwartzman reaction remains conjectural. The inflammatory process and the polymorphonuclear infiltration which follow the preparatory injection are, without doubt, determining factors. However, studies by Shwartzman(1,2) and P. Bordet(3) have emphasized that the specific reaction cannot be induced by any one inflammatory agent, and that endotoxins, originating from a variety of bacteria, retain in this respect a privileged position. Recent investigations(4) have shown the crucial role played by the circulating polymorphonuclear leukocytes; their presence is necessary for elicitation of the hemorrhagic reaction.

The purpose of the experiments to be described here was to investigate the role of certain intracellular enzymes of the protease type in the Shwartzman reaction. It was presumed that enzymes originating from the granules of the polymorphonuclear cells may be released or activated by endotoxins or other agents possessing similar properties.

The results reported below provide strong evidence in favor of such a conception. They indicate that, on the one hand, granules isolated from polymorphonuclear cells are able to induce regularly Shwartzman-type hemorrhagic tissue damage, as previously reported by Thomas(5,6), and that, on the other hand, a biological substance possessing potent antiprotease activity is capable of inhibiting regularly the Shwartzman reaction whether induced by endotoxins or by granules isolated from polymorphonuclear cells.

Materials and methods. Animals. Adult albino rabbits weighing between 3 kg to 3.5 kg, obtained from commercial sources, and fed with a stock laboratory diet, were used.

Hemorrhagic Shwartzman phenomenon. The local hemorrhagic reaction was induced by two different methods. In one group of 19 animals, the preparatory injection was performed either with purified endotoxin,* in a dose of 50 μg suspended in 0.5 ml of saline, or with the same volume of a filtrate of a 5-

* Endotoxin extracted from *E. coli* 026 B6, Difco Laboratories.

TABLE I. The Effect of a Protease Inhibitor (Zymofren = Trasylal) on the Shwartzman Reaction in Skin Prepared with Endotoxin.

Group	Treatment	Lesions: Size in mm	Lesions: Intensity of hemorrhage	Remarks
Preparatory injection: endotoxin	Controls	45/23	+++	Culture filtrate
		50/28	+++	" "
		30/40	+++	" "
		40/25	+++	" "
		60/30	++++	Endotoxin Difco
		35/40	++++	" "
		25/20	++	" "
	Antiprotease, 70,000 units	—	—	Culture filtrate
		—	—	" "
		—	—	" "
		—	—	Endotoxin Difco
		—	—	" "
		—	—	" "
		—	—	" "
		—	—	" "
		—	—	" "
		10/10	+	" "
		12/15	+	" "

The number of + indicates the intensity of hemorrhages.

day-old culture on meat broth of *E. coli*.† The eliciting injection was made in both cases, with 100 μg of purified endotoxin given intravenously 24 hours later.

In the second group composed of 13 animals, the skin was prepared by an intradermal injection of leukocyte granules, as described by Thomas(6); granules were isolated from washed granulocytes derived from peritoneal exudate according to the technique of Cohn and Hirsch(7). The animals were challenged by intravenous injection of 100 μg of purified endotoxin Difco given 5 hours later.

Preparation of granules from polymorphonuclear cells. The method used derives from those described previously(6,7). Rabbits weighing between 4 to 5 kg received intraperitoneally 500 ml of a 0.1% solution of glycogen in isotonic saline, followed 5 hours later by reinjection of 50 ml of isotonic saline containing 5 units/ml of heparin. The abdomen was gently massaged for a few minutes and the exudate drained through a large trocar set up in the peritoneal cavity. The exudate so obtained contained almost exclusively granulocytes.

The exudate was centrifuged at 1000 RPM for 30 minutes. The pellet containing the packed cells was washed 3 times with a 0.34 M sucrose solution and centrifuged, each time, 10 minutes at 1000 RPM. All procedures were carried out at 4°C.

The pellet collected after the last centrifugation was resuspended in cold sucrose and the volume adjusted to contain 10^9 cells/ml. The cells were then disrupted by drawing the suspension into a 20 cc syringe and expelling several times. Release of granules was indicated by a marked increase in turbidity and viscosity. The suspension was centrifuged at 800 RPM to remove cell debris, and the supernate containing granules was used for local skin preparation.

Protease inhibitor. The antiprotease used is the kallikrein inhibitor isolated by Frey, Kraut and Werle(8) from the beef parotid gland:‡ The substance has been identified as a polypeptide of about 11,500 M.W. The commercial preparation used here contains 1000 units/ml dissolved in isotonic saline.

The quantities injected varied from 70,000 to 82,500 units per animal and were administered as follows: 10,000 to 20,000 units were injected intravenously 15 minutes before the challenging injection and the remain-

† Strain from Pasteur Inst.

‡ The trade names of the protease inhibitor are "Zymofren," Specia, France; "Trasylal," Bayer, Germany.

TABLE II. The Effect of a Protease Inhibitor on the Shwartzman Reaction in Skin Prepared with Leukocyte Granules.

Group	Treatment	Lesions: Size in mm	Lesions: Intensity of hemorrhage	Remarks (after rubbing with alcohol)	
Preparatory injection with granules isolated from polymorphonuclear cells	Controls	10/20	++	50/50	+++
		15/20	++	30/50	++++
		20/25	+++	40/50	+++
		18/20	++	40/40	+++
		60/30	+++		
		30/40	+++		
		30/25	+++		
		15/15	—		
	Antiprotease, 82,500 units	—	—	—	
		—	—	+ some petechiae	
		—	—	+ "	"
		—	—	± "	"
		—	—	± "	"

The number of + indicates the intensity of hemorrhages.

der was given by intravenous perfusion, started immediately after the eliciting injection of endotoxin. The speed of the perfusion was adjusted to administer the whole dose of antiprotease within 3 hours.

The results were noted 3, 6, 12, 24 and 48 hours after the challenging injection. Tissue damage was evaluated by its macroscopic aspect, measurement of the 2 main diameters of the lesion, and degree of infiltration.

Results. The results are summarized in Tables I and II. It is apparent, from the data reported here and shown previously by Thomas(6), that granules isolated from polymorphonuclear cells and used as preparatory agent are capable of inducing hemorrhagic skin lesion with the same efficiency as endotoxin. An impression prevailed that lesions produced by the granules were more severe than those caused by endotoxin; however, more extensive studies are necessary for assessing such a statement. A shortening of the latent period was regularly possible when granules were used as preparatory injection instead of endotoxin.

The treatment with the antiprotease caused in almost all cases a remarkable inhibition of the hemorrhagic lesion, whether endotoxin or granules were used as the inducing agents. In the endotoxin group, all controls, with the exception of one, responded to the challenging injection with rather severe tissue damage. In this group of animals, the injection of the protease inhibitor provided full protection in 10 of 12 animals treated; in the 2 remaining, the lesion observed was considerably attenuated.

In the group of animals prepared with granules, the results were even more striking. While all the controls except one presented extensive, severe hemorrhagic and sometimes even necrotic lesions, the animals treated with the antiprotease were exempt from any visible tissue damage. Local rubbing with alcohol, which aggravates notably the hemorrhagic lesion and often makes it necrotic, only slightly affected the treated group.

Discussion. The salient fact which emerges from these findings is that pretreatment with a protease inhibitor prevents regularly and almost completely the local hemorrhagic Shwartzman phenomenon. The antiprotease used in these experiments is a kallikrein inhibitor isolated by Frey *et al*(8) from the beef parotid. It is a polypeptide of about 11,500 M.W. and it has no other known effect than the property of inhibiting strongly several proteolytic enzymes including trypsin, chymotrypsin, and plasmin. To the knowledge of the author, an inhibitory effect of this factor on the intracellular acid cathepsins has not been reported.

What may be the link between the antiprotease activity of the polypeptide and its protective effect against the tissue damage occurring in the Shwartzman reaction? This question should be considered in the frame of the general concept, recently formulated,

as to the role of the intracellular hydrolases (lysosomes) in the pathogenesis of inflammation(9,10) and of shock(11).

It has been hypothesized that the tissue damage in the Shwartzman reaction results from release or activation of intracellular hydrolytic enzymes released by endotoxin from the lysosomes of the polymorphonuclear cells(6).

It was previously shown that the presence of circulating polynuclears is essential for production of the Shwartzman reaction and that this reaction cannot be obtained in animals whose leukocytes have been previously destroyed by leukolytic agents.

Evidence of a release of lysosomal enzymes from hepatic cells during endotoxin and traumatic shock has been recently produced by Weissmann and Thomas(12). On the other hand, potentiation of the local Shwartzman reaction by trypsin has been reported by Antopol and Chryssanthou(13), who found also a certain degree of inhibition of the phenomenon by crude soybean trypsin inhibitor(14).

The data reported here support strongly the possibility that release of cathepsins may be the basis for tissue damage, by two arguments: 1) induction of the phenomenon by using polymorphonuclear leukocyte granules as preparatory injection; 2) the evidence that a substance possessing the exclusive property of inhibiting proteolytic enzymes inhibits the lesion.

The shortening of the latent period, when granules are used instead of endotoxin as preparatory factor, may be interpreted in favor of an indirect mechanism of action of endotoxins.

The action of the antiprotease on the Shwartzman reaction may share a common mechanism with the property of this substance to inhibit hemorrhagic pancreatitis(15, 16) which is another example of a hemorrhagic lesion produced by proteolytic enzymes.

If the local Shwartzman phenomenon is a miniature specimen of the generalized tissue damage occurring in various types of shock and in inflammation in general, the property of a natural, non-toxic factor of inhibiting such reactions takes a meaningful significance.

Summary. Severe local Shwartzman reactions have been induced in rabbits by endotoxin as well as by granules isolated from polymorphonuclear cells. Administration of polypeptide possessing potent antiprotease activity inhibited regularly and almost completely the Shwartzman reaction induced either by endotoxin or by polymorphonuclear granules. It is hypothesized that the tissue damage observed in the Shwartzman reaction is conditioned by release or activation of intracellular lysosomal enzymes contained in granulocytes.

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