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Effects in the Rat of Intradermal Injection of Purified Proteinases from Streptococcus and *Serratia Marcescens*[1] (39128)

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Complement as a mediator of the inflammatory response has been appreciated for several years (1–3); the various complement proteins are activated, enzyme complexes are formed, and small protein fragments are generated that have pathobiological activities including increased vascular permeability, smooth muscle contraction, and chemotaxis. More recently, attention has focused on nonimmune mechanisms of activation and cleavage of complement components. In addition to activities generated via the alternative or properdin pathway, it has been shown that certain proteolytic enzymes can act directly on complement components *in vitro* to produce fragments with activities identical or similar to those produced through the classical complement sequence (4).

Since an inflammatory response is characteristic of bacterial infections, we have investigated the possibility that proteolytic enzymes produced by bacteria might act directly on complement components *in vivo* to generate biologically active fragments. We have already shown that a purified proteinase from Group A, β-hemolytic streptococcus and a proteinase from *Serratia marcescens* can generate fragments from C3 and C5 *in vitro* which are chemotactic for polymorphonuclear leukocytes (5). In this paper, we report that these proteinases are potent permeability factors *in vivo* but the activity is expressed independently of the complement system.

Materials and methods. The following materials were kindly donated: Triprolidine hydrochloride (Actidil), Burroughs Wellcome Co., Research Triangle Park, North Carolina; methysergide maleate (Sansert), Sandoz Pharmaceuticals, East Hanover, New Jersey; Cyproheptadine HCl (Periactin HCl), Merck and Co., Inc., Rahway, New Jersey. *Serratia marcescens* proteinase was supplied by Dr. Charles Decedue, Department of Microbiology, Louisiana State University, Baton Rouge. It was purified by ammonium sulfate fractionation and DEAE cellulose chromatography of culture filtrates. Streptococcal proteinase was supplied by Dr. Stuart D. Elliott, prepared according to published procedures (6). Dr. Peter A. Ward supplied the rabbit anti-rat polymorphonuclear leukocyte serum and anti-MUBl (anti C5) serum, and Dr. Charles R. Annable supplied the rabbit anti-rat C3.

Both the streptococcal and *Serratia* proteinases were diluted in phosphate buffered saline, pH 7.4, the former also containing 0.01 *M* 2-mercaptoethanol. Proteolytic activity of both enzymes was determined by a modification (6) of the Kunitz assay for trypsin (7), using casein as a substrate. In some experiments, the enzymes were inactivated by heating: 90° for 30 min for the streptococcal proteinase and 56° for 10 min for the *Serratia* proteinase.

Cobra venom factor (CoF) was prepared from lyophilized cobra venom (*Naja naja*, Ross Allen Reptile Institute, Inc., Silver

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Springs, Florida) by DEAE cellulose chromatography (8) and used without further purification. Albino rats weighing about 200 g were injected intraperitoneally with 10 units of partially purified CoF in a volume of 0.5 ml at 4 to 6-hr intervals over a 24-hr period, so that a total of 50 units were administered. On one occasion, a group of animals received 250 units of CoF/kg body wt in four doses over a 48-hr period. Immediately prior to the experiment, blood samples were taken for titrating the residual hemolytic activity (CH_{50}) and levels of C3 and C5 by radial immunodiffusion.

Measurements of the permeability increasing activity of the bacterial proteinases and the effects of pharmacological antagonists were performed as described previously (2, 9). All experiments were performed in 175–225 g albino rats previously injected intravenously with Pontamine Sky Blue. In some cases the rats were anesthetized with sodium nembutal (Abbott Laboratories, North Chicago, Illinois) (4 mg/100 g body wt). Results with nembutal-treated and untreated animals were indistinguishable. In some experiments, skin sites were excised, fixed in buffered formalin, paraffin-sectioned, and stained with hematoxylin and eosin.

Rats were depleted of polymorphonuclear leukocytes by the intraperitoneal injection of 2 ml of rabbit antiserum about 18 hr prior to the experiment. The antiserum had been adsorbed with rat erythrocytes and lymphocytes and depleted circulating polymorphonuclear leukocytes to less than 500/mm^3. On the day of the test total cell counts and reduction in neutrophils were determined for each animal.

Results. Vascular permeability in rat skin increased in a dose response fashion following intracutaneous injection of 0.1 ml solutions of increasing amounts of streptococcal proteinase or *Serratia* proteinase (Fig. 1). Bluing occurred rapidly, was maximal within 10 min, and persisted at least 24 hr. Positive reactions were observed with <1 μg of streptococcal proteinase and 2 μg of *Serratia* proteinase; nearly maximal bluing occurred at 10–20 μg of either enzyme. At very high concentrations (>25 μg for the streptococcal enzyme and 200 μg for the *Serratia* enzyme) both proteinases produced hemorrhagic necrosis within 10–20 min after injections. Heated enzymes that were inactive in the casein assay did not increase permeability.

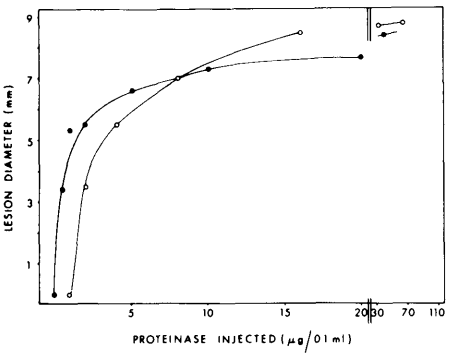

FIG. 1. Effect of intravenous injections of bacterial proteinases on increased vascular permeability in rat skin. Streptococcal proteinase: ●—●; *Serratia* proteinase ○—○.

Microscopic examination of skin from animals injected 3 hr previously with 25 μg of streptococcal proteinase showed necrosis of epidermal tissue, lifting off of the keratinized layer, dermal inflammation, and edema and infiltration of polymorphonuclear leukocytes into the subcutaneous fat and muscle. Results were similar at 6 and 12 hr with the additional appearance of mononuclear cells. At 24 hr the cellular infiltrate was predominately mononuclear with deposition of fibrin and early regeneration of the epidermis also noted (Fig. 2). At concentrations of the proteinases which gave only bluing (e.g. 5 μg), the histological responses were similar but temporally delayed. Epithelial necrosis was not detected earlier than twelve hours after injections.

Effect of various treatments on permeability activity of the bacterial proteinases. 1. Cobra factor treatment. The possible role of terminal components of complement on the above *in vivo* responses was investigated in rats treated with CoF. Depletion of C3 levels to less than 4% of normal levels, as measured by hemolytic activity, did not alter the diameter of the bluing compared with control animals. The C3 levels in rats receiving five times the recommended dose

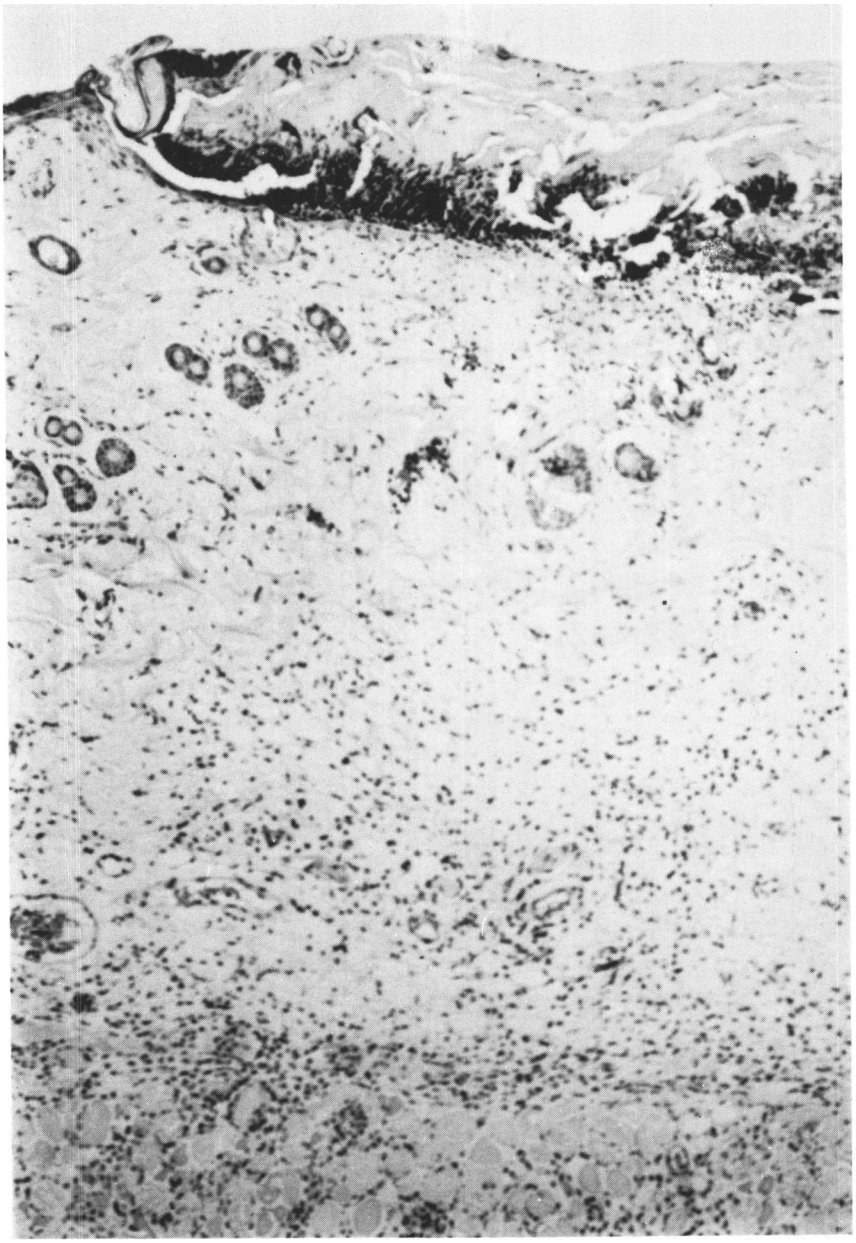

FIG. 2. Section of rat skin excised 24 hr after injection of 25 μg of streptococcal proteinase showing infiltration of mononuclear cells, fibrin deposition, and early regeneration of epidermis. Hematoxylin and eosin; $\times$ 200.

of CoF were still about 4% of the controls, and data from both these experiments are included in Table I. Histologic sections of lesions from the CoF-treated animals were indistinguishable from those of normal animals.

2. Intravenously injected tripolidine, methysergide, and cyproheptadine. To determine whether the enzymes were acting through release of histamine or serotonin, lesion diameters were compared before and after intravenous injection of antagonists of

TABLE I. EFFECT OF COMPLEMENT DEPLETION ON ENHANCED VASCULAR PERMEABILITY

Micrograms injected in 0.1 ml	Lesion diameter (mm)	
	CoF-treated[a]	Buffer controls
Streptococcal proteinase		
100	8.8[b]	10.7[b]
33	9.5[b]	7.5[b]
5	6.6	6.8
2.5	7.5	7.3
1.3	6.0	5.2
0.6	4.8	3.8
Serratia proteinase		
1000	11.7[b]	10.8[b]
200	10.7[b]	9.5[b]
40	6.8	8.8
8	6.0	7.3
1.6	4.0	3.8

[a] CoF-treated rats had < 4% residual CH_{50}, 2% residual C3 and < 2% residual C5 by radial immunodiffusion as compared with buffer controls.

[b] Hemorrhagic necrosis accompanied lesion.

these mediators. There was no inhibition of the responses to either bacterial proteinase after administration of the antihistamine tripolidine in an amount (0.5 mg/kg body wt) that completely abolished the response to 50 μg histamine (Table II). The serotonin antagonist methysergide (1 mg/kg), which inhibited permeability caused by 5 μg of serotonin, also inhibited threshold doses of streptococcal proteinase but had no effect on the reaction to *Serratia* proteinase. Cyproheptadine (1 mg/kg) inhibited the reaction to both 50 μg of histamine and 5 μg of serotonin, and also inhibited the lower concentrations of streptococcal proteinase but not the *Serratia* enzyme.

3. Depletion of polymorphonuclear leukocytes. The bluing response of polymorphonuclear leukocyte-depleted rats to intracutaneous injections of streptococcal proteinase was the same as the response of control animals. Histologic examination of lesions in the depleted animals about 6 hr after injection of enzyme showed a loss of cohesion between cells, loosening and necrosis of the epithelium, but no corresponding inflammatory reaction. Mast cells were

TABLE II. EFFECT OF INTRAVENOUSLY INJECTED ANTIHISTAMINE AND ANTISEROTONIN ON ENHANCED VASCULAR PERMEABILITY

Micrograms injected in 0.1 ml	Lesion diameter (mm)			
	Control	+Tripolidine[a]	+Methysergide[b]	+Cyproheptadine[b]
Streptococcal proteinase				
5	6.6[c]	6.5	6.8	5.3
2.5	6.3	5.9	5.3	2.9
1.25	4.5	4.9	4.7	0.6
0.63	4.0	4.8	0.8	0
0.31	1.3	0	0	0
Serratia proteinase				
16	8.6[d]	7.8	8	7.9
8	7.3	7.4	7	7.1
4	5.4	5.0	5.8	5.5
2	3.7	3.3	3.6	3.5
Histamine (50 μg)	10	0	ND[e]	0
Serotonin (5 μg)	11	ND[e]	0	0

[a] 0.5 mg/kg.

[b] 1 mg/kg.

[c] Each diameter average of six to 10 animals.

[d] Each diameter average of four animals.

[e] Not done.

more prominent and more numerous than in controls. By 24 hr there was a considerable amount of necrosis, a few cells that could be distinguished as polymorphonuclear leukocytes, and much cellular debris (Fig. 3). Control sites injected with 0.01 *M* mercaptoethanol (diluent) showed none of the above reactions. One control and one depleted animal were injected intravenously with bovine serum albumin (BSA) and subcutaneously with anti-BSA. As expected, the reversed passive Arthus reaction was completely inhibited in the animals depleted of polymorphonuclear leukocytes (10).

Discussion. Since the demonstration that purified bacterial proteinases can generate phlogistic products from purified complement components *in vitro* (4), it has been tempting to speculate that complement-derived products might be important in the

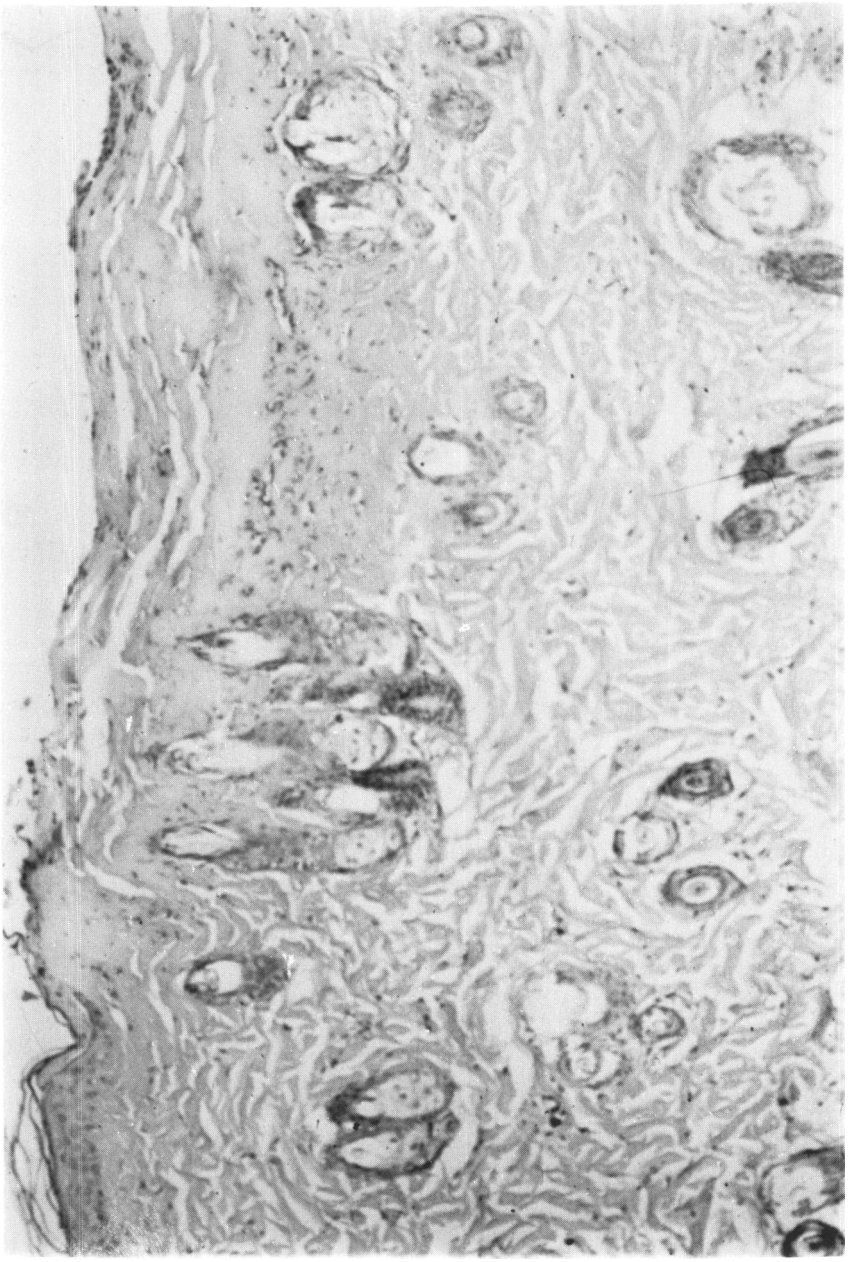

FIG. 3. Section of skin from neutrophil-depleted rat excised 24 hr after injection of 5 μg of streptococcal proteinase showing necrosis, a few polymorphonuclear leukocytes, and cellular debris. Hematoxylin a id eosin; × 200.

in vivo inflammatory response associated with various microorganisms (4, 11). Indeed, on the basis of preliminary *in vitro* work, Gazzinelli *et al.* (12) have suggested that complement might be implicated in the pathogenesis of *Schistosoma* infections. Incubation of partially purified extracts from *S. mansoni* cercariae produced in whole rat or guinea pig serum an activity similar to the anaphylatoxin derived from C5 (C5a). However, although the two purified bacterial proteinases we have studied proved to be potent permeability factors *in vivo*, and generated chemotactic factors from C3 and C5 *in vitro*, they in fact acted independently of complement and histamine, and further, did not depend on the presence of neutrophils to sustain tissue injury.

Although we could detect no evidence that histamine was important in the inflammatory response caused by either bacterial proteinase, there was some inhibition of lesions produced by lower concentrations of streptococcal proteinase in the presence of serotonin antagonists. Whether this suggests a difference in mechanism of action of the enzymes or is merely quantitative is not clear. Neither cyproheptadine nor methysergide had any effect on the caseinolytic activity of streptococcal proteinase *in vitro*.

Summary. Purified streptococcal proteinase and *Serratia* proteinase are potent permeability factors in rat skin and initiate histopathological evidence of an acute inflammatory response. These effects appear to be largely independent of terminal components of complement, histamine, and polymorphonuclear leukocytes.

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