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Identification of Staphylococcal Panton-Valentine Leukocidin as a Potent Dermonecrotic Toxin

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The Panton-Valentine leukocidin of *Staphylococcus aureus* was shown to exhibit a potent dermonecrotic effect when injected intradermally into rabbits. This effect could be abrogated by immunizing animals with the F component or both components, but immunization with the S component appeared to exacerbate certain of the intradermal responses.

The necrotizing activity of intradermal injections of toxigenic staphylococci or cell-free staphylococcal culture supernatant is well established (2, 5). The dominant feature of many of these lesions is an ischemic necrosis considered to be caused by staphylococcal alpha-toxin (3, 4). Two other staphylococcal hemolysins, beta and delta, have been shown to be dermonecrotic, but their activity is several orders of magnitude less than that of alpha-toxin (12, 15).

In our work on the role of toxins and antitoxins in the pathogenesis of staphylococcal mastitis, we investigated the Panton-Valentine leukocidin (PVL). This toxin consists of two individually inactive components, F and S, which together exhibit the toxic properties of PVL (19). The action of PVL on leukocytes is precisely defined (19), but its in vivo action has received scant attention. If it is injected systemically into rabbits, it provokes a granulocytopenia followed by a marked granulocytosis (11), but its intradermal activity has not been investigated. Woodin commented that PVL "was not a very toxic substance" (19).

In the experiments reported here, the effects of injecting purified PVL and its components intradermally into control rabbits and into rabbits specifically immunized with either of the components or whole PVL were investigated.

MATERIALS AND METHODS

Panton-Valentine leukocidin. (i) For immunizing. Purified F and S components of PVL isolated from culture filtrates of *Staphylococcus aureus* strain V8 were obtained in crystalline form from the late A. M. Woodin. Both components were inactive when tested in vitro with human polymorphonuclear leukocytes in the presence of known active heterologous component. In a pilot experiment both were shown to have retained their antigenicity.

(ii) For challenge. Active components for intradermal challenge were purified from standard material supplied by Microbiological Research Establishment, Porton Down, Salisbury, England (8). The materials as supplied were effectively free from active heterologous component but were not pure. At one stage, purification by isoelectric focusing in a gradient (pH 7.5 to 10), stabilized by sucrose in an LKB 8101 column (LKB, Bromma, Sweden), was used. The components (300 units of each) were dialyzed overnight against 1% glycine. They were then loaded onto the column together with a cocktail of ampholytes, giving a final concentration of 1.5% ampholyte. The power applied initially was 6 W, and as the current fell, the voltage was increased over 30 h to a maximum of 1,100 V. After 44 h at 4°C, the column was emptied, and 1-ml fractions were collected. After pH measurements at 4°C, the fractions were neutralized. They were assayed for: (i) both leukocidin components by human polymorphonuclear leukocytes; (ii) hemolysins by twice washed rabbit, sheep, and horse erythrocytes; and (iii) the following activities by standard methods—tributyrinase (14), egg yolk factor (lecithinase) (16), fibrinolysin (17), free coagulase (7), phosphatase (7), deoxyribonuclease (13), and gelatinase (9). Fractions showing only the appropriate leukocidic activity were pooled, dialyzed over 48 h against three changes of 1,000 volumes of 0.05 M PO_4 buffer (pH 7.4), and assayed. This material was stored frozen until used, and immediately before use it was reassayed. No material was kept in this purified state for more than 7 days.

Assay of PVL components and antibodies. The two components were assayed by a semimicro-modification of the method described by Woodin (18). Each component was assayed in the presence of excess of the other component. The method for the F component will be described briefly; for the S component the letters F and S should be interchanged.

Serial 20% dilutions of the material to be assayed were prepared in phosphate-buffered saline ($\mu = 0.144$, pH 7.2) + 0.5% gelatin, and 25 μl of each dilution was placed in microtiter trays. Excess of the S component contained in 25 μl of phosphate-buffered saline-gelatin was then added to each well, followed by 25 μl of antiserum (EX4573; Wellcome Research Laboratories, Beckenham, England) diluted in phosphate-buffered saline-gelatin to contain 0.5 U of antibody per ml against the F component. These mixtures were left at room temperature for 20 min, and then 10 μl from each well was transferred to monolayers of human polymorphonuclear leukocytes. The monolayers were prepared by allowing fresh human blood to clot on

clean areas of a prepared glass slide and then removing the clot (10). After addition of the toxin-antitoxin mixtures to the monolayers, they were incubated in a moist atmosphere at 37°C for 20 min. Subsequently, they were read by phase-contrast microscopy, and the titer was expressed as a range between the last well, to show swollen, granular, nonrefractile polymorphonuclear leukocytes, and the first, to show intact viable cells. All titers were expressed in international units per milliliter.

Antitoxins were titrated by a modification of the method of Gladstone et al. (10); the method for anti-F antibodies will be described: 25-μl doubling dilutions of the antiserum were prepared in phosphate-buffered saline-gelatin in microtiter trays, and 25 μl of a toxin mixture containing 0.2 U of F per ml and 2.0 U of S per ml in phosphate-buffered saline-gelatin was added. After standing at room temperature for 20 min, 10 μl from each well was transferred to human polymorphonuclear leukocyte monolayers prepared as for toxin assay. After 20 min at 37°C, they were read microscopically, and the titer was expressed as a two-fold range in international units per milliliter.

Immunization and challenge. Groups of four rabbits of approximately 2.5 kg (Hyline Rabbits Ltd., Marston, Northwick, England) were injected with either individual F or S components or a mixture of equal amounts of both. The crystalline material supplied by Woodin was used without toxoiding, as all toxic activity had been lost.

The separate components were made up at 200 μg per ml in 0.05 M phosphate buffer (pH 7.2) and treated for 2 min in an ultrasonic generator (MSE, Crawley, England) at low power. The F component dissolved completely, but the S component remained as a fine suspension. Both were emulsified with equal volumes of Freund complete adjuvant (Wellcome), and 0.5 ml containing 50 μg of antigen was injected into each thigh muscle of the respective rabbits. Animals receiving both components received them in separate injections as above.

A second injection of 25 μg of antigen in Freund complete adjuvant was given 33 days later, and on days 35, 36, and 37, 25 μg of antigen adsorbed onto alhydrogel (25% wt/vol) (Superfos Export Co., Copenhagen, Denmark) was given intravenously in 0.1 ml.

Animals were bled on day 40, and the sera were assayed. They were challenged intradermally on day 42 with 5 U, 2 U, 1 U, and 0.5 U of each component or mixtures in 0.2 ml of phosphate-buffered saline plus 1% normal rabbit serum. Lesions were read at 2 h, 24 h and 48 h. High toxin doses were separated to avoid overlap, and all rabbits received both the individual component and the mixtures.

Passive neutralization of toxic effects. Doubling dilutions of a rabbit antiserum containing equal amounts of antibody to F and S, starting at 16 U/ml, were prepared. An equal volume of an equimolar mixture of the F and S components was added, and the mixtures stood at room temperature for 30 min. A 0.2-ml amount of each sample was then injected intradermally into rabbits. Lesions were read at 40 h.

RESULTS

Purity of toxin. (i) Immunizing antigens. The material obtained from A. M. Woodin was inactive in all the biological tests used; therefore, purity was checked by immunodiffusion of antisera raised against the two components and a crude culture supernatant known to contain many antigens, including both leukocidin components.

As can be seen from Fig. 1, the antisera raised against either component reacted with only one purified component but sometimes gave a fuzzy second line with the crude material (Fig. 1A, wells 1 and 4). This is probably due to antibody to the heterologous component, in this case anti-S antibody, which was present in relatively high concentrations in the serum used (Table 1).

In further tests, the antisera contained no neutralizing antibody to α- or β-hemolysins, suggesting that the immunizing antigens were free of these toxins in immunogenic forms.

Challenge toxin. Isoelectric focusing of the

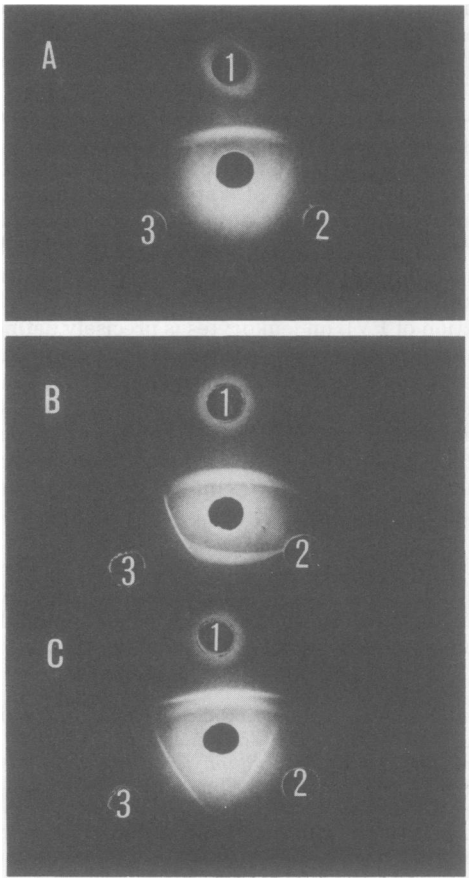

FIG. 1. *Immunodiffusion of antisera versus crude PVL and purified F and S components. Well 1, crude PVL; well 2, purified F; well 3, purified S; well 4, antiserum. A, Anti-F antiserum, rabbit 130; B, anti-S antiserum, rabbit 133; C, anti-F and anti-S antisera, rabbit 134.*

Porton material gave purified components with isoelectric points for the F component in the range 9.2 to 9.4 and for the S component 8.9 to 9.2. These materials were free from hemolysin, tributyrinase, lecithinase, fibrinolysin, free coagulase, phosphatase, and gelatinase, but contained very low levels of deoxyribonuclease. Each component showed leukocidin activity only when combined with the other component, and this activity was neutralized by specific antisera.

Response to immunization. Each of the individual components raised antibodies against both the F and S components. The titer against the immunizing agent was always greater than that against the heterologous component (Table 1).

Response to challenge. Control. The data are summarized in Table 2. Two hours after challenge, all of the injection sites, including the control of 1% normal rabbit serum, showed mild localized inflammation. By 24 h the control mark and marks associated with all doses of the S component had faded. With the exception of the top dose, marks due to the F component had also faded; the top dose, however, produced small areas (1 cm^2) of mild erythema. Mixtures of both components had by this time provoked areas of inflammation, subcutaneous hemorrhage, and edema. Doses as low as 0.5 U of each component produced this acute response. By 48 h the lesions associated with mixtures of >1 U of each component had developed necrotic centers, and the erythema and edema seen at lower doses had intensified (Table 2). In contrast, F-associated lesions had, by this time, resolved.

Passive neutralization of the lesions produced by mixtures of equal amounts of F and S is shown in Fig. 2. Neutralization of the effect of a mixture of 2.5 U of each component is shown at top right, and the severity of the lesion increases from top right through bottom left.

(ii) F-immunized. By 24 h the initial inflammation had subsided, and by 48 h a faint blush marked the injection sites of >1 U of the mixed components. No other lesions were visible (Table 2).

(iii) S-immunized. After showing mild localized inflammation at 2 h, these rabbits had, by 24 h, developed significant reactions. Both individual components produced dose-dependent areas of erythema ranging from 0.5 cm to 2 cm in diameter. The response to the S component was only marginally less than that to the F component. This is in contrast to the result in control animals where the S component was without apparent effect. Mixtures of the two components gave lesions similar to but less intense than those given in control animals (Table

TABLE 1. *Anti-PVL antibody levels of experimental rabbits*

Rabbit no.	Immunizing antigen	Antibody titer (U/ml^{-1})	
		Anti-F	Anti-S
127	F	6–12	1.6–3.2
129	F	6–12	1.6–3.2
130	F	>12	3–6
132	S	1.6–3.2	6–12
133	S	3–6	>12
134	F + S	>12	>12
135	F + S	>12	>12
136	F + S	>12	>12
139	—[a]	0.8–1.6	0.8–1.6
140	—	0.4–0.8	0.4–0.8

[a] —, None.

TABLE 2. *Response of rabbits to intradermal inoculation of PVL components*[a]

Intradermal PVL dose (U)		Control		F immunized		S immunized		F + S immunized	
F component	S component	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
5	—[b]	2 E	1 E	Tr	1 E±	3 E+	3 E++ S	Tr	1 E
2	—	1 E±	1 E±	Tr	Tr	2 E+	2 E+ S	Tr	1 E
1	—	1 E±	1 E±	Tr	Tr	2 E	2 E+ S	Tr	1 E
0.5	—	0	0	Tr	Tr	1 E	1 E	Tr	0
—	5	E±	0	Tr	Tr	2 E S	3 E+ S	Tr	1 E
—	2	0	0	Tr	Tr	1 E S	2 E++ S	Tr	1 E
—	1	0	0	Tr	Tr	1 E	1 E+ S	Tr	1 E
—	0.5	0	0	Tr	Tr	1 E	1 E+	Tr	0
5	5	3 E++ SN	3 E++ SN	Tr	Tr	3 E+ S	3 E++ SN	Tr	2 E
2	2	2 E++ SN	2 E++ SN	Tr	Tr	3 E+	2 E+ S	Tr	1 E
1	1	2 E+ S	2 E++ S	Tr	Tr	2 E S	2 E S	Tr	0
0.5	0.5	2 E+ S	2 E+ S	Tr	Tr	2 E S	1 E+	Tr	0

[a] Size of lesion on a scale of 0 to 3: 0 = no trace; Tr = trace; 1 = <1 cm diameter; 2 = 1 to 4 cm diameter; 3 = >4 cm diameter; E to E++ = intensity of erythema; S = edema; N = necrosis.

[b] —, None.

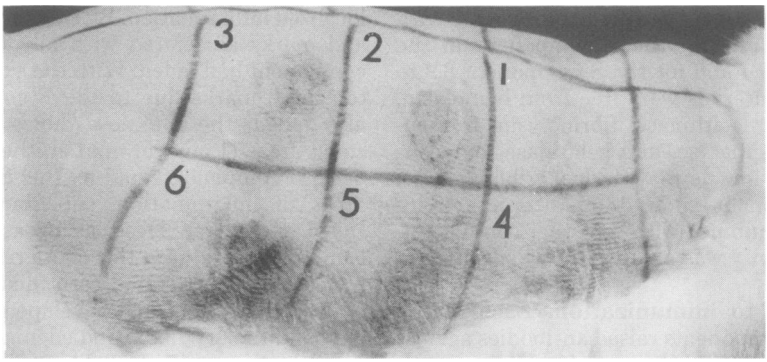

FIG. 2. *Passive neutralization of the effect of 2.5 U of F plus 2.5 U of S. A constant amount of toxin was incubated with doubling dilutions of an antiserum containing equal neutralizing titers to both F and S. The antibody concentration falls from site 1 to site 6.*

2). By 48 h all the lesions had become more intense and swollen.

(iv) F- and S-immunized. The response in these animals was similar to that in the F-immunized group. The S component provoked no visible reaction, whereas the highest doses of both the F component and the mixtures produced mild erythema.

DISCUSSION

It is known that PVL is produced in staphylococcal infections of both rabbits and humans, for anti-PVL antibodies have been found in animals or patients with chronic infections (1, 10). The relevance of the action of this toxin in either the establishment or progression of staphylococcal infections is, however, not yet known. The results presented here indicate that when its role is considered further, both its well documented leukocidic action and a potent dermonecrotic action must be taken into account. The severity of the lesions produced by intradermal injection of 5 U of the mixed components was surprising. They were comparable with those produced by 10 μg of staphylococcal alpha-toxin (16). As no values for the specific activity of PVL in units per microgram are available, a direct comparison between these results cannot be made.

Neutralization of the dermonecrotic effect by antibodies against the F component or both components can be understood if it is assumed that, as with leukocidic activity, the dermonecrotic effect is shown only when both components are present in an active form. The apparent enhancement of the effect of the F and S components and the lack of neutralization of the effect of the mixed components in rabbits immunized with the S component are, on this basis, surprising. Sera from these rabbits are able to neutralize the leukocidic activity of active mixtures, e.g., in the antibody assay. However, when these lesions are compared with those seen in unimmunized or F-immunized animals, the histological appearance is quite different. The lesions seen in S-immunized animals presented a picture of mononuclear, as opposed to polymorphonuclear, cell infiltration, suggesting that this response is a cell-mediated response and that the lesions are due to delayed-type hypersensitivity.

It was of interest to note that both individual components gave rise to antibodies against both components. Two possible explanations of this are (i) that the components were contaminated with low levels of inactive homologous component, or (ii) that there is a degree of immunological cross-reactivity between the two components. The first of these is unlikely since in a test to determine the total combining power of the two components in each "pure" material, a value of 0.2 U/ml (the limit of the assay) was obtained for the heterologous component. The second alternative seems the most likely.

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