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Comparison of Subcutaneous and Intraperitoneal Staphylococcal Infections in Normal and Complement-Deficient Mice

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From a comparison of the effects produced by injecting different strains of *Staphylococcus aureus* either subcutaneously or intraperitoneally into normal, complement-deficient, or complement-depleted mice, it was possible to assess the pathogenic significance of various staphylococcal virulence factors and the defensive role of complement components in the two sites of infection. In skin lesions the inflammation-suppressing factor found in the cell walls of strain PS80 played a major role. In contrast, in intraperitoneal infection the antiphagocytic capsule of the Smith diffuse and M strains was more important. All strains used produced alpha-hemolysin, which is the ultimate lethal agent in intraperitoneal infection but is only one factor in the production of dermonecrosis. The severity of the skin lesions was inversely related to the amount of early fluid exudate rather than to the rate of bacterial growth, whereas in the peritoneum increased bacterial growth was associated with increased mortality. Both C3 and C5 were needed in the production of fluid exudate in response to staphylococcal skin infection. C3 appeared to be more important in the increased exudate formed in immune mice. In the peritoneum the opsonic and chemotactic actions for complement were important as shown by the results in cobra venom-treated normal mice and in C5-deficient B10D2 old-line mice.

The pathogenic effect of a specific microorganism depends on the strain used, the species and strain of host involved, and the dose and route of infection. The "virulence" of a pathogen can only be defined by using these terms together with a clear indication of the way in which virulence is measured.

The interaction of these factors can be illustrated by comparing intraperitoneal and subcutaneous staphylococcal infections in mice.

The intraperitoneal injection of *Staphylococcus aureus* into mice stimulates the accumulation of polymorphonuclear leukocytes (PMN) in the peritoneal cavity. PMN provide the main defense against infection by this route since they phagocytose and kill the staphylococci (11). The phagocytosis of noncapsulated staphylococci requires the presence of either complement or immunoglobulin, whereas both these factors are needed to opsonize those strains with an antiphagocytic capsule (11). This requirement is reflected in the greater virulence of capsulated strains of *S. aureus* in the nonimmune mouse (11). They resist phagocytosis, multiply rapidly, and produce alpha-hemolysin, which kills the mouse. Protection requires specific immunoglobulin M opsonins (8). Antitoxic antibodies delay but do not prevent death (12). Noncapsulated strains are readily phagocytosed and destroyed, although if the initial dose is very high the phagocytes may be overloaded.

In contrast, subcutaneous *S. aureus* infection produces a localized lesion, the severity of which may be enhanced by the presence of a foreign body such as a cotton dust plug (16). Using this method, the virulence of the *S. aureus* is assessed by the degree of dermonecrosis, which is in turn inversely related to the extent of early fluid exudation in the lesion (2, 3). Dermonecrosis has been attributed to the action of alpha-hemolysin (3, 16), but there is evidence to suggest that a specific cell-mediated response is also involved (7). Protection against dermonecrosis is antibody mediated and is closely related to rapid early fluid exudation at the site of infection (6). The severity of dermonecrosis varies according to the *S. aureus* strain used. "Virulent" strains have the ability to delay the onset of fluid exudation (2). Hill (9) isolated a mucopeptide protein complex (DOCR) from the residue left when cell walls of

virulent strains were extracted with sodium deoxycholate. DOCR from virulent strains inhibited early fluid exudation, enhanced the severity of *S. aureus* skin lesions, and inhibited leukocyte chemotaxis in vitro, whereas DOCR from avirulent strains did not (9, 23).

Serum complement is involved in opsonization and phagocytosis, chemotaxis, and the vascular changes of acute inflammation (14, 19, 20, 24) and should therefore play an important part in both routes of *S. aureus* infection in the mouse.

In this paper we have compared the effects of *S. aureus* strains in subcutaneous and intraperitoneal infection in mice, using dermonecrosis and death, respectively, as measures of virulence. We have also examined in vivo the effect of complement depletion on these infections, using cobra venom factor and zymosan to deplete the third component of complement (C3) and mice genetically deficient in the fifth component (C5).

MATERIALS AND METHODS

Mice. Female outbred Wright-Fleming Institute (WFI) mice weighing 20 to 25 g were used. Female B10D2 old-line and B10D2 new-line mice of the same weight were also used to study the effect of C5 deficiency. These two mouse strains are co-isogenic. The old-line mice lack C5, whereas the new-line mice have a normal complement system (5). These particular strains have been used previously to study the role of C5 in experimental bacterial infections (14, 19).

Bacteria. Strain M (NCTC 10649) is a heavily capsulated strain of *S. aureus* described by Scott (18). The capsulated Smith diffuse and noncapsulated compact types have been widely used in studies on intraperitoneal infection in mice (8). PS80 (NCTC 9788) and Wood 46 (NCTC 10345) are noncapsulated strains that have been used frequently for subcutaneous *S. aureus* infection (1, 6).

All strains were stored freeze-dried and when reconstituted were maintained on nutrient agar slopes at 4 C.

Production and assessment of intraperitoneal infection. *S. aureus* strains from overnight cultures in 0.1% glucose digest broth were washed twice in saline and injected in 0.5 ml of saline. The dose given was judged by the optical density of the suspension and confirmed by viable counts. Mice were observed for 1 week, although most deaths occurred within 48 h. In experiments on preopsonization, bacteria were injected suspended in fresh normal mouse serum. For bacterial counts mice were killed 6 h after infection and the peritoneum was washed out with saline containing 10 U of heparin per ml. Leukocytes were disrupted by ultrasonication at a level that had no effect on staphylococcal viability. This treatment also served to break up larger clumps of staphylococci. Smears of the peritoneal washings were prepared and stained with Giemsa. Leukocytes in the exudates were counted in a hemocytometer.

Production and assessment of subcutaneous infection. Five microliters of a suspension of *S. aureus* in saline adjusted to contain 10^5 to 3×10^5 colony-forming units (CFU) was absorbed onto a cotton dust pellet at the end of a wide-bore needle. The needle was inserted subcutaneously and the cotton pellet was expelled. Before injection, the mice were shaved and depilated with Nair. Skin lesions were examined 24 h after infection and scored as follows. Dermonecrosis was expressed as a necrotic index (NI) where $NI = \Sigma(Dd)/n$. D and d are major and minor diameters, respectively, of the necrotic area in millimeters; n is the number of mice infected.

The skin lesions were excised with a circular punch. The fluid exudation was assessed by the increase in weight of the skin with the lesion over that of a piece of normal skin from the same mouse (1). The skin was then cut into small pieces and homogenized for 3 min in 10 ml of saline, using a Colworth stomacher (A. J. Seward & Co. Ltd., Bury St. Edmonds). This is a laboratory blender in which a threshing action is applied to material in a sealed polyethylene bag. Viable counts were then done on 10-fold dilutions of the extract, using nutrient agar (Oxoid).

Immunization. Mice receiving intraperitoneal infections were immunized by the intraperitoneal injection of 10^9 heat-killed homologous organisms in saline 3 and 2 weeks before challenge. Immunization was equally possible with smaller doses (10^7 CFU) of live organisms, but there were occasional deaths and it seemed preferable to use killed bacteria. Mice challenged subcutaneously were given two doses of 10^5 CFU of living *S. aureus* subcutaneously on cotton dust 1 week apart and were used 10 to 14 days after the second injection. In the subcutaneous infection no immunity was produced by dead organisms.

Complement depletion. Hemolytic complement was measured by the method of Rosenburg and Tachibana (17), where the lysis of optimally sensitized labeled sheep erythrocytes by mouse serum was assessed by the release of ^{51}Cr (Radiochemical Centre, Amersham). No hemolytic activity was detected in serum from B10D2 old-line mice. C3 was measured by radial immunodiffusion in agar containing 10% C3 antiserum. This antiserum was raised in New Zealand white rabbits by the repeated injection of zymosan-C3 complexes in Freund complete adjuvant (Difco) (13). Since C3 standards were not used, this technique only measured C3 levels in complement-depleted mice relative to those in untreated animals.

In vivo complement depletion. Cobra venom factor (Cordis) was injected intraperitoneally in two doses of 100 U/kg over 24 h. Mice were used 24 h after the second injection. Zymosan (Koch Light) was suspended in saline at a concentration of 10 mg/ml. Mice were given 2 mg intraperitoneally.

RESULTS

Comparison of S. aureus strains injected subcutaneously on cotton dust in WFI mice. The subcutaneous injection of 10^5 CFU of *S. aureus* Smith diffuse or M on cotton dust pro-

duced mild non-necrotic skin lesions similar to those produced by *S. aureus* Wood 46 but unlike the severe dermonecrotic lesions that followed the injection of *S. aureus* PS80. The capsulated staphylococcal strains stimulated more early fluid exudation than did PS80 (Table 1), but despite the presence of antiphagocytic capsules multiplied no faster in the skin than did the noncapsulated types (Table 2). These results suggest that *S. aureus* Smith diffuse and M lack active DOCR. The presence of a capsule can mask certain activities of *S. aureus* such as that of bound coagulase. The fact that the Smith compact strain also produced mild lesions makes it seem unlikely that the capsule of *S. aureus* Smith diffuse was masking the effects of an active DOCR.

Effect of zymosan and cobra venom factor on complement levels in mice. Zymosan induced a rapid fall in both hemolytic complement and C3 levels (Table 3). By 24 h, however, there was no detectable fall in C3 and hemolytic activity was normal or almost so. Cobra venom factor, however, produced a greater and more prolonged depletion, C3 and hemolytic complement levels not returning to normal for 72 h. As used neither agent affected circulating levels of leukocytes. Zymosan has been shown to have no effect on the functions of murine PMN (21).

Effect of complement depletion on S. aureus skin infection in immune and nonimmune WFI, B10D2 new-line and B10D2 old-line mice. The subcutaneous injection of 10^5 CFU of *S. aureus* PS80 on cotton dust produced dermonecrosis in noninfected WFI, B10D2 new-line, and B10D2 old-line mice, although the lesions were consistently larger in B10D2 old-line mice than in the other two strains (Tables 4 and 6). The injection of a similar dose of *S. aureus* into mice immunized by previous infection only produced mild erythematous lesions, the B10D2 old-line mice being equally protected against the dermonecrosis. In each case the degree of dermonecrosis was inversely related to the amount of early fluid exudation in the lesion. C3 depletion with zymosan or cobra venom factor enhanced the extent of dermonecrosis and reduced early fluid exudation both in the normal and previously infected mice of all strains (Tables 4 and 6). In all these experiments we found no difference in bacterial growth rates (Tables 5 and 7). Zymosan was only effective during the time in which it depleted C3 and hemolytic complement levels (Table 3). When given 24 h before challenge with *S. aureus* PS80 it had little effect on the severity of skin lesions or on early fluid exudation (Tables 4 and 6). Cobra venom factor, as expected, was still effective after 24 h.

TABLE 1. *Skin lesions and 4-h fluid exudation in WFI mice after the subcutaneous injection of 10^5 CFU of S. aureus strains on cotton dust*

S. aureus strain	No. of mice with necrosis (group of 12)	Necrotic index	Increased skin wt (mg)
PS80	12	30	27
Wood 46	2	2	48
Smith diffuse[a]	0	0	45
Smith compact	0	0	40
M[a]	0	0	50

[a] Capsulated strain.

TABLE 2. *Bacterial counts in WFI mice after the subcutaneous injection of 10^5 CFU of S. aureus strains on cotton dust*

Time after infection (h)	Counts × 10^5			
	PS80	Smith diffuse[a]	M[a]	Wood 46
0	0.8	0.6	0.6	0.6
2	6	4	6	3
4	10	20	30	20
6	50	60	50	90
24	2,000	1,500	3,000	2,000

[a] Capsulated strain.

TABLE 3. *Effect of zymosan and cobra venom factor (CoVF) on complement levels in WFI, B10D2 old-line,[a] and B10D2 new-line mice*

Treatment	CH_{50} level[b]		Reduction (%) in C3		
	WFI	B10D2 new	WFI	B10D2 old	B10D2 new
None	55	30	0	0	0
Zymosan, 4 h[c]	16	<5	65	70	50
Zymosan, 24 h	44	32	0	0	0
CoVF, 24 h	<10	<5	<80	60	80
CoVF, 48 h	<10	12	<80	60	70
CoVF, 72 h	53	27	0	<10	<10

[a] No hemolytic activity was detectable in any B10D2 old-line mice.
[b] CH_{50}, 50% hemolytic complement.
[c] Time between administration of agent and measurement of complement.

TABLE 4. *Effect of zymosan and cobra venom factor (CoVF) on skin lesions and 4-h fluid exudation in normal and previously infected WFI mice after the subcutaneous injection of 10^5 CFU of S. aureus PS80 on cotton dust*

Mice	Zymosan	CoVF	Mice with necrosis (group of 12)	Necrotic index	Increased skin wt (mg)
Noninfected			12	25	30
Noninfected	1 h[a]		12	81	10
Noninfected	24 h		12	33	28
Noninfected		24 h[a]	12	45	15
Infected			0	0	88
Infected	1 h		12	11	28
Infected	24 h		3	2	75
Infected		24 h	9	8	27

[a] Time between administration of agent and injection of *S. aureus*.

TABLE 5. *Effect of zymosan and cobra venom factor (CoVF) on bacterial counts in the lesions of WFI mice after the subcutaneous injection of 10^5 CFU of S. aureus PS80 on cotton dust*

Time after injection (h)	Bacterial counts/lesion ($\times 10^5$)		
	Control	Zymosan	CoVF
0	0.5	0.5	0.6
2	3	4	6
4	30	20	40
6	90	50	100
24	1,000	700	2,000

S. aureus Wood 46 caused little dermonecrosis and considerable fluid exudation when injected subcutaneously on cotton dust. Treatment with zymosan or cobra venom factor enhanced the severity of infection with *S. aureus* Wood 46, presumably by inhibiting the early fluid response. We could not, however, enhance the severity of *S. aureus* Smith diffuse in this way (Table 8).

Complement and intraperitoneal infection in mice with S. aureus. Table 9 shows the effect of zymosan and cobra venom factor on the intraperitoneal injection of various strains of *S. aureus* into nonimmune WFI mice. As expected, the capsulated *S. aureus* strains Smith diffuse and M produced a high mortality with relatively few organisms. As noted by Scott (18) and Melly et al. (15), *S. aureus* M was more virulent than Smith diffuse. The noncapsulated strains PS80 and Wood 46 did kill mice if the dose injected was raised to 10^9 CFU. C3 depletion enhanced the virulence of all strains tested except Smith compact.

WFI mice were protected against capsulated strains by prior immunization with heat-killed homologous staphylococci. Zymosan and cobra venom factor still enhanced the virulence of the challenge strains, showing that both complement and immunoglobulin opsonins were needed for complete protection. Replacement of complement by incubating the challenge organisms in fresh normal mouse serum reduced the mortality in complement-depleted immune WFI mice, but not to the levels seen in animals with a normal complement system (Table 10).

B10D2 old-line mice were more susceptible than the new line to challenge with Smith diffuse and M (Table 11).

The mortality of nonimmune mice challenged with *S. aureus* Smith diffuse was related to the growth of bacteria in the peritoneal cavity (Fig. 1). Treatment with cobra venom factor resulted in more rapid growth than in control mice. Even with Smith compact, mice treated with cobra venom factor killed the organisms more slowly than did normal controls. The growth of Smith diffuse was more rapid in B10D2 new-line than in old-line mice (Fig. 2).

The normal mouse has a mononuclear cell population in its peritoneal cavity. Two hours after the injection of 10^8 CFU of *S. aureus* Smith diffuse PMN began to appear in the peritoneal washings, and by 6 h 90% of cells were PMN. In mice treated with cobra venom factor the increase in numbers of PMN was unaffected for the 2 h after infection but then slowed (Fig. 3).

DISCUSSION

The *S. aureus* strains PS80 and Smith diffuse or M are at opposite ends of the spectrum with regard to their effect on mice after subcutaneous or intraperitoneal infection. *S. aureus* Wood 46 occupies an intermediate position. PS80 produced severe dermonecrotic skin lesions but on intraperitoneal injection only killed mice in high doses. Smith diffuse and M strains produced mild skin lesions but severe intraperitoneal infections. Despite these differences, both dermonecrosis and death have been attributed to alpha-hemolysin, which is produced by all three strains. The antiphagoytic

TABLE 6. *Effect of zymosan and cobra venom factor (CoVF) on skin lesions and 4-h fluid exudation in normal and previously infected B10D2 old- and new-line mice after the subcutaneous injection of 10^5 CFU of S. aureus PS80 on cotton dust*

Mice	Zymosan[a]	CoVF	Mice with necrosis (group of 12)		Necrotic index		4-h increase in skin wt (mg)	
			Old line	New line	Old line	New line	Old line	New line
Noninfected	−	−	12	12	48	27	10	25
Noninfected	+	−	12	12	75	64	8	12
Noninfected	−	+	12	12	103	83	6	14
Infected	−	−	0	2	0	2	70	83
Infected	+	−	9	6	15	12	24	30
Infected	−	+	8	10	12	17	31	33

[a] Administered 1 h before *S. aureus* challenge.

TABLE 7. *Bacterial counts in B10D2 old- and new-line mice after the subcutaneous injection of 10^5 CFU of S. aureus PS80 on cotton dust*

Time after injection (h)	Bacterial counts (CFU)/lesion ($\times 10^5$)	
	Old line	New line
0	0.7	0.6
2	4	2
4	20	40
6	800	700
24	1,000	2,000

capsule of Smith diffuse and M strains is the major virulence factor in the peritoneum (11, 12) but from our experiments appears to confer no advantage to the pathogen in the skin. Active DOCR appears to play a lesser role in the peritoneum, though as Agarwal (2) and Hill (9) showed, it is critical in delaying the early inflammatory response in the skin.

In the mouse, complement is important in both subcutaneous and intraperitoneal infection with *S. aureus*. The activation of complement splits C3 into a large (C3b) and small (C3a) fragment. C3a has anaphylotoxic activity, releasing histamine from mast cells, and is also chemotactic.

C3b is an opsonin (19) but also splits a low-molecular-weight fragment C5a from C5. C5a, like C3a, causes histamine release and is chemotactic for PMN (20). The importance of C5b as an opsonin remains to be fully defined, but it does seem to be necessary for the optimal phagocytosis of pneumococci in vitro (19). The PMN that are attracted by C3a and C5a release a variety of lysosomal contents that split more C3 and C5 and thus intensify the reaction (22).

As already reported (14), there is less early exudate in skin lesions in C5-deficient than in normal mice. The slight further decrease in exudate in C5-deficient mice after zymosan or cobra venom factor suggests that C3 may also play a part. The more marked effect of these reagents in normal mice could be due to C5 depletion sequential to C3 activation. Comparison of early exudation in immunized old- and new-line mice with and without zymosan or cobra venom factor suggests that the C5 contribution persists but that most of the immune exudate is due to C3. We have suggested that the accelerated early fluid response to subcutaneous *S. aureus* infection seen in immunized mice is an Arthus reaction resulting from complexes of staphylococcal antibody and antigen (6). Crisler and Frank (Fed. Proc. 24: 447, 1967) have shown that C5-deficient B10D2 old-line mice can give a normal Arthus reaction. We have not yet examined the rates of fluid exudation in peritoneal infections.

Agarwal (2) noted the early accumulation of PMN in subcutaneous skin lesions in immune mice, but there was no evidence that the killing of bacteria by PMN affected the dermonecrotic

TABLE 8. *Effect of zymosan and cobra venom factor (CoVF) on skin lesions after the subcutaneous injection of 10^5 CFU of S. aureus Wood 46 and Smith diffuse on cotton dust into nonimmune WFI mice*

S. aureus strain	Mice with necrosis (group of 12)			Necrotic index		
	No treatment	CoVF	Zymosan	No treatment	CoVF	Zymosan
Wood 46	1	4	6	1.5	8	14
Smith diffuse[a]	0	0	0	0	0	0

[a] Capsulated strain.

TABLE 9. *Mortality in nonimmune WFI mice after the intraperitoneal injection of S. aureus strains in saline*

S. aureus strain	Dose	Treatment[a]	Mice dead (group of 20)	
			No.	%
Smith diffuse[a]	10^8		20	100
	10^7		6	30
	10^7	CoVF[b]	18	90
	10^7	Z	18	90
M[b]	10^7		20	100
	10^6		0	
	10^6	CoVF	18	90
	10^6	Z	18	90
Smith compact	10^9		0	
	10^8		0	
	10^8	CoVF	0	
	10^8	Z	0	
PS80	10^9		8	40
	10^8		0	
	10^8	CoVF	20	100
	10^8	Z	12	60
Wood 46	10^9		14	70
	10^8		0	
	10^8	CoVF	15	75
	10^8	Zymosan	10	50

[a] CoVF, Cobra venom factor; Z, zymosan.
[b] Capsulated strain.

TABLE 10. *Mortality in immune WFI mice after the intraperitoneal injection of capsulated S. aureus strains in saline; effect of preopsonisation with fresh mouse serum*

S. aureus strain	Dose	Treatment[a]	Preopsonisation	Mice dead (group of 20)	
				No.	%
Smith diffuse[b]	10^8		−	6	30
		CoVF	−	16	80
		CoVF	+	10	50
		Z	−	14	70
		Z	+	9	45
M[b]	10^7		−	2	10
		CoVF	−	17	85
		CoVF	+	9	45
		Z	−	14	70
		Z	+	8	40

[ab] See Table 9.

skin lesion (6). Complement depletion consistently failed to increase the bacterial counts in *S. aureus* skin lesions. Again events in the peritoneal cavity were different. The capsulated Smith diffuse strain grew to a greater extent in C5-deficient old-line than in the normal new-line mice. Both Smith diffuse and compact strains grew better when normal WFI mice were treated with cobra venom factor. Increased bacterial growth in mice was associated with higher mortality. Our results suggest that C5 and probably also C3 were acting as opsonins for the capsulated strains. They may also have been acting as chemotactic factors, but we do not yet have sufficient data to quantitate the relative importance of the C3a and C5a in these two processes in staphylococcal peritoneal infection. Cobra venom factor did not to-

TABLE 11. *Mortality in B10D2 old- and new-line mice after the intraperitoneal injection of capsulated S. aureus strains in saline*

S. aureus strain	Dose	Mice dead (group of 20)			
		Old line		New line	
		No.	%	No.	%
Smith diffuse[a]	10^7	12	60	8	40
	5×10^6	8	40	1	5
	10^6	0	0	0	0
M[a]	5×10^6	20	100	15	75
	10^6	16	80	3	15
	5×10^5	0	0	0	0

[a] Capsulated strain.

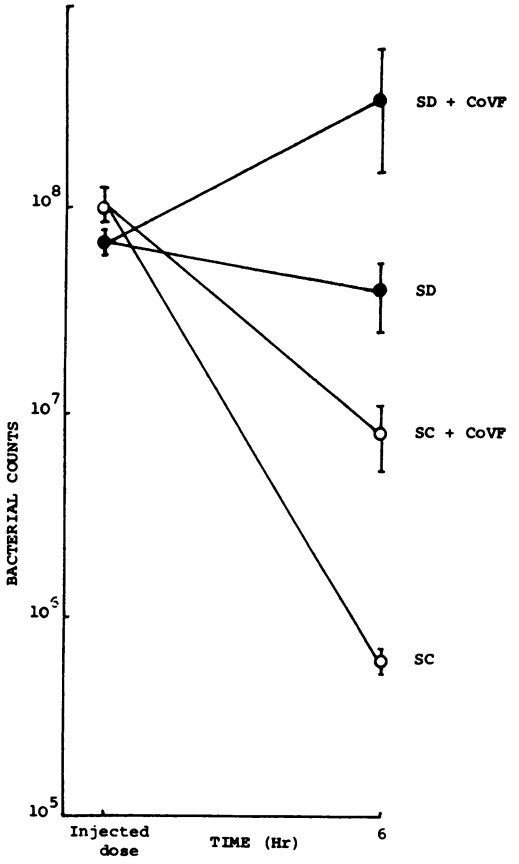

FIG. 1. *Viable bacterial counts in peritoneal fluid of WFI mice after the injection of S. aureus Smith diffuse (SD) and compact (SC) strains. Effect of cobra venom factor (CoVF).*

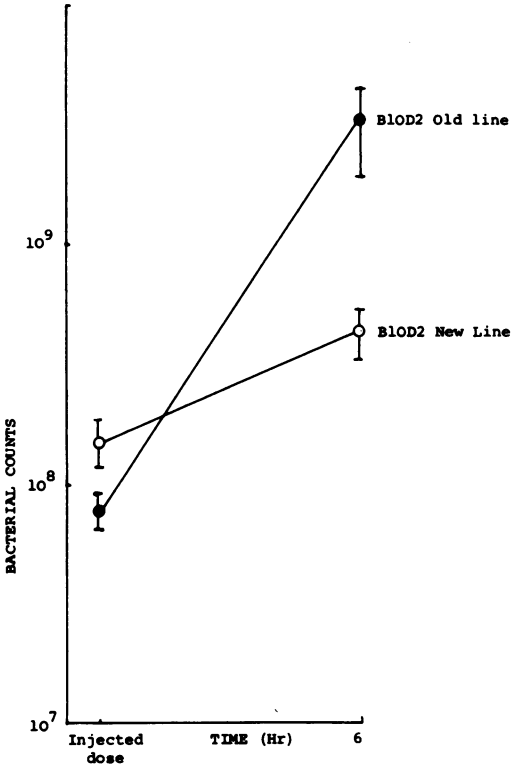

FIG. 2. *Viable bacterial counts in peritoneal fluid of B10D2 old- and new-line mice after the injection of S. aureus Smith diffuse strain.*

tally prevent the accumulation of PMN in the peritoneum, but only lowered the numbers of cells after the first 2 h. There was residual C3 in the mice treated with cobra venom factor, but complement-independent chemotactic mechanisms may also have been working.

In man C3 deficiency has been associated with recurrent pyogenic infections (4) but not with *S. aureus*. Jacobs and Miller, however, have described recurrent *S. aureus* infections in infants with a C5 abnormality (10).

The two experimental models of infection we have examined show the importance of not drawing general conclusions about the pathogenesis of disease from one route of infection, even in the same species. No one animal model may be directly relevant to human staphylococcal disease, but each may reveal different facets of the interaction of *S. aureus* with host defenses that need to be considered in relation to the range of staphylococcal infections in man. Yet another problem is that of the staphylococcal abscess, which might be regarded as a more satisfactory lesion than necrosis to study in the skin. Mice do develop staphylococcal abscesses, but their pathogenesis in relation to the lesion so far studied needs further analysis.

ACKNOWLEDGMENTS

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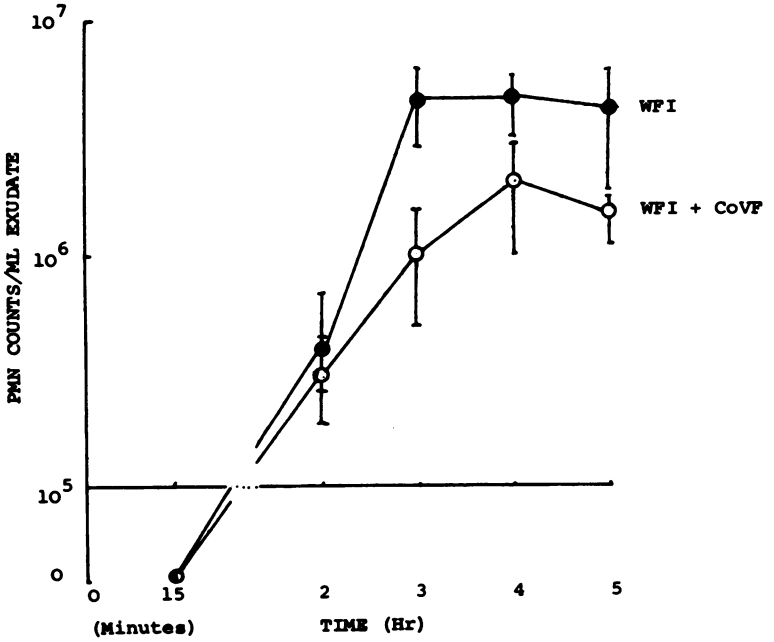

FIG. 3. *Effect of cobra venom factor (CoVF) on PMN counts in peritoneal fluid of WFI mice after the injection of 10^8 CFU of S. aureus Smith diffuse.*

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