FACTORS RELATING TO THE VIRULENCE OF STAPHYLOCOCCI

II. Observations on Four Mouse-Pathogenic Strains*

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A series of studies designed to determine the factors which characterize virulence of staphylococci have been carried out in this laboratory over the past several years. In a recent paper it was shown that the pathogenicity of a variant of the Smith staphylococcus was due to its resistance to phagocytosis in the mouse peritoneum (1). This strain (the "diffuse" variant) showed a number of unusual properties not shared by most strains of staphylococci isolated from human infections. It grew in long, comet-shaped colonies in soft agar containing serum or plasma; it lacked "clumping factor" or "bound" coagulase (1); it required both heat-stable antibody and heat-labile factors for opsonization (2). Simultaneous studies by Morse showed that the Smith staphylococcus possessed a distinctive surface antigen inhibiting phagocytosis (3).

It was thus our initial belief that this microorganism represented an atypical staphylococcus—a biologic sport of doubtful relationship to the problem of staphylococcal virulence for man. Subsequent studies have caused us to consider an alternative hypothesis. Recently, Tompsett reported observations on 4 staphylococcal strains of high mouse virulence which resembled the Smith diffuse variant in lacking clumping factor (4). The present paper examines the biologic characteristics of these 4 additional clumping factor–negative staphylococcal strains and compares them with the diffuse variant of the Smith staphylococcus previously described.

The data suggest that these 5 strains share the same or similar phagocytosis-inhibiting antigens and that this property accounts for their virulence in the peritoneal cavities of mice. Indirect evidence leads us to postulate that the

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properties of these unusual strains may offer clues to the more important problem of *in vivo* virulence of staphylococci in man.

Materials and Methods

Cultures.—The 5 mouse-virulent strains and 4 coagulase-positive strains isolated from human infection were studied simultaneously. The mouse-virulent strains were the Smith diffuse variant, previously described (1) and 4 other clumping factor–negative strains kindly supplied by Dr. Ralph Tompsett. These included Welwood, a strain isolated from a routine vaginal culture and Adams, a strain isolated from a routine throat culture at Baylor University Medical Center. The K-6 and K-93 strains were both originally described by Alami and Kelly (5). K-6 was isolated from the nasopharynx of a healthy carrier; K-93 was obtained from a cutaneous ulcer.

Three strains of *Staphylococcus aureus* isolated from human infections observed in patients at Vanderbilt Hospital within the last year were employed as typical human pathogens. All produced free coagulase and clumping factor as well as alpha and delta hemolysins. These included the Bowers strain, isolated from a patient with an infected vascular graft, Jackson, isolated from the blood stream of a patient with staphylococcal septicemia, and Herrod, isolated from the pleural space of a patient with staphylococcal empyema.

The Giorgio strain has been previously utilized in laboratory studies here and elsewhere (6). It produced free coagulase and clumping factor as well as alpha and delta hemolysins.

Demonstration of Hemolysins.—Quantitative alpha hemolysin titrations were performed as previously described (1). The plate methods of Gillespie and Simpson (7) and Elek and Levy (8) were employed to determine hemolysin patterns. Filter paper strips saturated with staphylococcal antitoxin,[1] 20 units per ml, were used as a source of antihemolysin.

Studies on Coagulase Production.—Quantitative coagulase studies were performed by the method of Yotis and Ekstedt (9). Bound coagulase or clumping factor was determined as described by Elek (10).

Penicillin Susceptibility.—The penicillin sensitivities of staphylococci were carried out in beef heart infusion broth using standard twofold tube dilution techniques. One-half ml of a 10^4 dilution of an 18 hour culture of the microorganism to be tested was used as the inoculum.

Phage Typing.—Phage typing was performed with routine test dilutions by the Tennessee Department of Health Laboratory in Nashville. Phage susceptibility of the clumping factor–negative strains was also independently studied by Dr. John Blair at the Hospital for Joint Diseases in New York City. In addition to testing at routine test dilutions (RTD) and at 1000 times RTD, the strains were also grown in broth for 4 hours at 45°C and these cultures were used to inoculate typing plates for repeat testing at RTD and 1000 times RTD in an effort to bring out phage patterns of non-typable strains. When no lysis was noted, the strains were again grown at 45°C, exposed to 55°C for 5 minutes, and then used to inoculate other plates. In addition to the standard series of phages, 6 additional phages, (42B, 47C, 52B, 69, 73, and 78) were used by Dr. Blair.

In Vitro Opsonic Studies.—The opsonic requirements of staphylococci were studied in phagocytic systems employing human leukocytes as previously described (2). At appropriate intervals, samples were removed from rotating siliconed tubes, coverslip smears prepared, stained with Wright's stain, 100 polymorphonuclear leukocytes counted, and the percentage of polymorphonuclear cells participating in phagocytosis enumerated.

Quantitative Determinations of Culturable Intraperitoneal Staphylococcal Populations.—Male Swiss albino mice weighing approximately 20 gm, obtained from Dublin Laboratory Animals,

[1] Obtained from Medical Research Council, National Institute for Medical Research, London, and United States Public Health Service, National Institutes of Health, Bethesda.

Inc., Dublin, Virginia, were used in all experiments. Mice were infected by intraperitoneal injections of 0.4 ml of 18 hour trypticase soy broth cultures of the staphylococcal strain under study. The inoculum was determined by plate counts at each experiment and was designed to contain at least 10^8 viable units. Immediately after infection and at 6 hours, groups of 3 mice were sacrificed, and quantitative bacteriologic counts of total staphylococci, extracellular staphylococci, and staphylococci associated with leukocytes were determined on peritoneal fluids as previously described (1). In the final calculations, results were pooled and averaged in the construction of figures.

Preparation of Staphylococcal Vaccines and Immunization of Mice.—Heat-killed, whole cell vaccines were prepared as in previous experiments (1). These vaccines contained no detectable alpha hemolysin before or after heat inactivation. Mice received 0.1 ml of vaccine every 3 or 4 days for a total of 5 doses intravenously *via* a tail vein. Animals were challenged by intraperitoneal administration of the strain under study 10 to 12 days after completion of immunization.

TABLE I

Survival of Mice Following Intraperitoneal Infection

Strain	No. of organisms injected	Deaths per No. of mice	Time of death
			hrs.
Clumping factor–negative strains			
Smith diffuse	3.8×10^8	9/10	6 to 8
Welwood	4.8×10^8	9/10	6 to 20
Adams	5.3×10^8	9/10	11 to 21
K-6	2.8×10^8	9/10	9 to 21
K-93	2.9×10^8	9/10	13 to 21
Clumping factor–positive strains			
Giorgio	5.1×10^8	0/10	—
Herrod	5.4×10^8	0/10	—
Jackson	1.2×10^8	0/10	—

RESULTS

Mortality Associated with Intraperitoneal Infection with Clumping Factor–Negative and Clumping Factor–Positive Strains.—As shown in Table I, the 4 clumping factor–negative strains behaved like the Smith diffuse variant. All produced fatal disease when injected intraperitoneally in doses of 2.8 to 5.3×10^8 viable units. Deaths occurred somewhat later with 3 strains than in the Smith diffuse variant, although the Welwood strain killed the majority of mice within 8½ hours. As with the diffuse variant infection, all mice dying succumbed within the first 24 hours. In contrast, 3 strains isolated from human infections which produced clumping factor did not cause fatal intraperitoneal infection when injected in similar numbers. All mice injected with these strains survived a 28 day observation period.

Biologic Characteristics of the Clumping Factor–Negative Strains.—When

grown in infusion broth, trypticase soy broth, tryptose phosphate broth, or brain heart infusion broth, all of the clumping factor–negative strains exhibited typical staphylococcal turbidity. They produced typical staphylococcal colonies on infusion agar or human blood agar plates and elaborated variable amounts of golden pigment.

In contrast to most pathogenic strains, the clumping factor–negative strains produced diffuse, streaming colonies when grown in serum or plasma soft agar media at 37°C for 36 hours (11). The appearance of these long, comet-shaped colonies was consistently delayed in cultures of the Adams strain. Seventy clumping factor–positive strains of staphylococci recently isolated from human specimens all produced compact ball-like colonies in plasma soft agar.

The clumping factor–negative strains all fermented mannitol, were inhibited by 0.1 μg/ml of penicillin G or less, produced free coagulase, and alpha and delta hemolysins. None of these strains was phage-typable at routine test dilutions as performed in the Tennessee Department of Health Laboratory or at 1000 times RTD using special techniques as performed by Dr. John Blair.

In contrast, the bound coagulase-positive strains utilized in the present studies were all phage-typable at routine test dilutions. Their phage patterns were as follows: Bowers, 53/83A; Jackson, 75; Herrod, 80/81 and Giorgio 47/53/54/75/77.

Table II summarizes the biologic characteristics of the 5 atypical strains and their quantitative coagulase and alpha hemolysin titers.

Opsonic Requirements of Clumping Factor–Negative Strains.—Previous studies have shown that the Smith diffuse strain requires both a heat-stable serum antibody, and heat-labile serum factor for opsonization in fluid systems (2). The results of similar studies on the present strains are summarized and compared with simultaneous results obtained with the Smith diffuse strain and 2 clumping factor–positive staphylococci isolated from human infection in Table III. These experiments were performed in a system containing washed human leukocytes suspended in 20 per cent adult human serum, normal rabbit serum, or serum from rabbits immunized with heat-killed diffuse variant vaccine.

As noted in Table III, 3 out of the 4 clumping factor–negative strains required both heat-stable serum factor resembling antibody and heat-labile factor resembling complement for opsonization. The heat-stable factor could be supplied by human serum or immune rabbit serum, but not by normal rabbit serum. Heat-labile factor could be supplied by fresh unheated human serum, unheated sera from normal or immune rabbits, or guinea pig complement. As previously noted, other pathogenic staphylococci, such as the Herrod and Bowers strains, were less resistant to phagocytosis, and ingestion occurred in the presence of *either* heat-stable *or* heat-labile serum factors (2). The Adams strain appeared to occupy a middle ground. While it was not opsonized by

TABLE II

Characteristics of Clumping Factor–Negative Strains

Test	Smith diffuse	Welwood	Adams	K-6	K-93
Source	Osteo	Normal vagina	Normal throat	Normal throat	Cutaneous ulcer
Phage type	NT	NT	NT	NT	NT
Growth in soft agar	Diff (++++)	Diff (+++)	Diff (++)	Diff (+++)	Diff (++++)
Free coagulase	1:256	1:64	1:32	1:16	1:16
Clumping factor (bound coagulase)	0	0	0	0	0
Mannitol	+	+	+	+	+
Hemolysins–					
alpha	1:64	1:32	1:64	1:128	1:64
delta	+	+	+	+	+

TABLE III

Opsonic Requirements

Serum	Clumping factor–negative strains					Clumping factor–positive
	Smith diffuse	Welwood	Adams	K-6	K-93	Herrod
Rabbit						
Diffuse immune rabbit serum....	++++*	++++	++++	++++	++++	++++
Heated immune serum.....	0	+	0	0	0	++
Normal rabbit serum.....	0	+	++++	+	+	++++
HIRS + NRS‡...	++++	++++	++++	++++	++++	++++
						Bowers
Human						
Human serum.....	++++	++++	++++	++++	++++	++++
Heated human serum.....	0	+	+	0	0	+++
Guinea pig complement.....	0	+	+++	+	+	++++
HHS + GPC§....	++++	+++	++++	+++	++++	++++

* Indicates degree of phagocytosis at 30 minutes.

‡ Heated immune rabbit serum plus normal rabbit serum.

§ Heated human serum plus guinea pig complement.

heated serum which contained antibody but lacked heat-labile factors, it was readily ingested in the presence of normal rabbit serum or guinea pig complement.

The Dynamics of Intraperitoneal Infections in Normal and Immunized Mice.— Normal mice and mice immunized with heat-killed vaccines prepared from the Smith diffuse variant and the Giorgio strain were challenged intraperitoneally with each of the clumping factor–negative strains. The results of these experi-

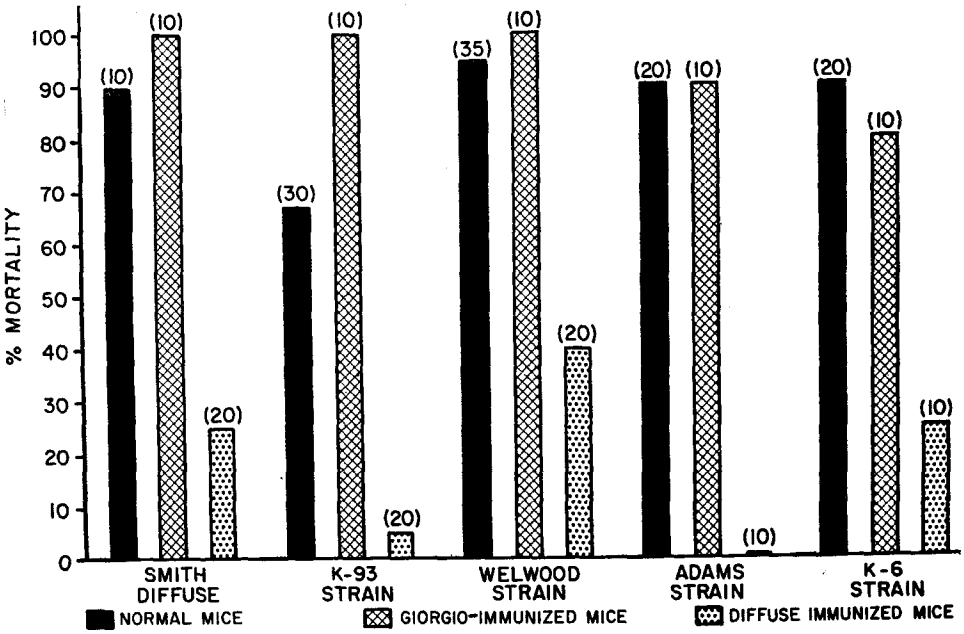
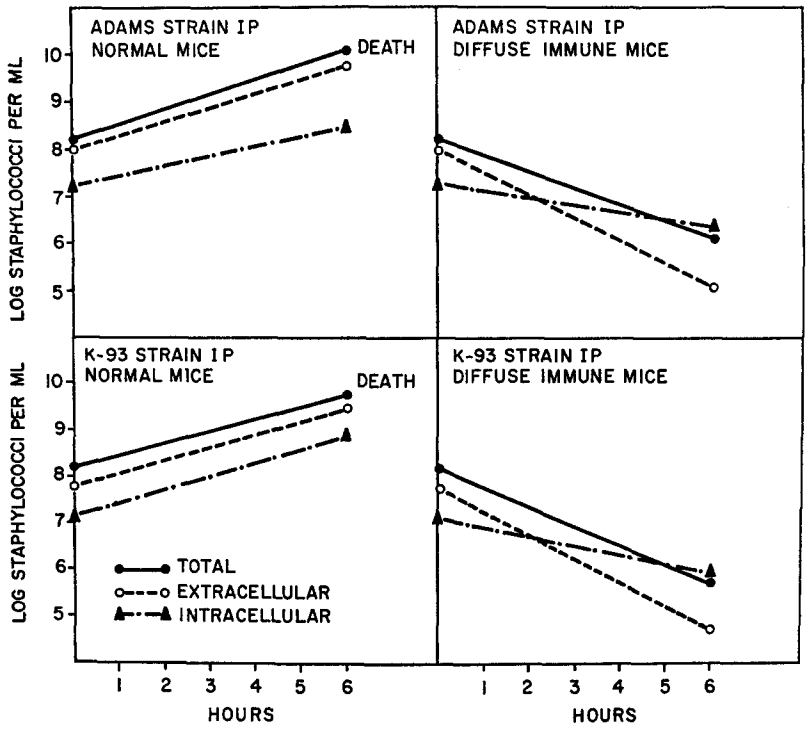


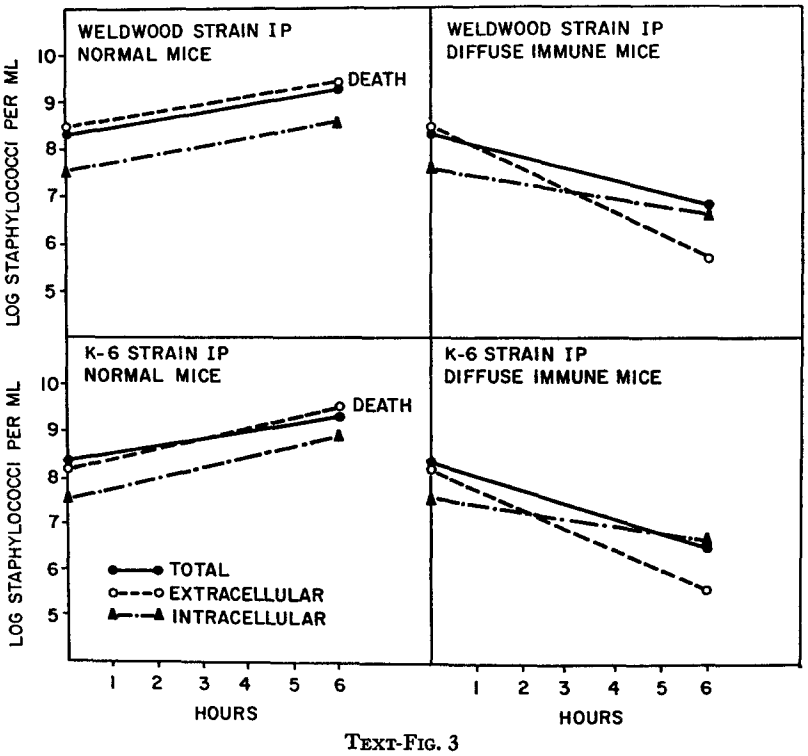
FIG. 1. Mortality of normal and immunized mice challenged with clumping factor–negative staphylococci. The number of mice in each group is indicated at the top of each bar.

ments are shown in Fig. 1. As noted here, 67 to 95 per cent of the normal mice died following challenge with 1.7 to 8.6 $\times$ 10^8 viable units of any of the 4 strains. Mice immunized with diffuse variant vaccine showed definite and striking protection to similar intraperitoneal challenge, mortality ranging from 0 to 40 per cent in these animals. In contrast, immunization with the clumping factor–positive strain, Giorgio, conferred no apparent protection and produced no significant change in mortality rates.

Quantitative studies performed during the course of the intraperitoneal infection revealed striking differences in the cellular management of these strains in normal mice and mice immunized with the diffuse variant vaccine. As noted in Figs. 2 and 3, all of the clumping factor–negative strains were



TEXT-FIG. 2



TEXT-FIG. 3

FIGS. 2 and 3. Quantitative bacterial studies on the intraperitoneal behavior of clumping factor–negative staphylococci in normal and diffuse variant immunized mice.

resistant to phagocytosis in the peritoneal cavity. Total microbial populations rose progressively, viable microorganisms remained extracellularly, and such animals died when intraperitoneal titers of 10^9 to 10^{10} were attained. In contrast, these strains were readily ingested in the peritoneum in diffuse variant immunized mice. The culturable extracellular staphylococci fell sharply as the total populations of viable microorganisms declined, indicating that phagocytosis was occurring. Such mice survived infection. Similar results were obtained in previous studies on diffuse variant immune mice challenged with the diffuse variant staphylococcus (1).

DISCUSSION

The current studies indicate that these unusual strains of *Staphylococcus aureus* closely resemble the Smith diffuse variant. All produce fatal intraperitoneal infections in mice when injected in numbers at which most strains isolated from human disease produce no significant mortality. All yield diffuse, streaming colonies when grown in soft agar containing plasma or serum. All lack the property of clumping when placed in plasma in slide coagulase studies. Three of the 4 required the presence of both heat-stable and heat-labile serum factors for opsonization. In contrast, strains of clumping factor–positive staphylococci previously studied have appeared less specific in their opsonic requirements (2).

From their behavior in the peritoneal cavities of mice, it appears that the virulence of these strains is closely related to their resistance to phagocytosis. The fact that immunization with a heat-killed vaccine prepared from the diffuse variant promotes brisk intraperitoneal phagocytosis and confers striking protection suggests: (*a*) that they have surface antigens identical with or closely related to the Smith diffuse variant; (*b*) that virulence among these strains is dependent on a surface structure or capsule retarding phagocytosis rather than extracellular enzymes or toxins. Further evidence to support this belief derives from the observations that vaccine without demonstrable alpha hemolysin activity is protective, and that most strains which produce similar or greater amounts of coagulase and alpha hemolysin are avirulent for mice in this experimental model.

These observations have suggested that these unusual strains may be tentatively separated from the majority of coagulase-positive staphylococci of human infection as schematically portrayed in Fig. 4. The mouse avirulent, clumping factor–positive strains which constitute the majority of staphylococci isolated from human material, while sharing certain common surface antigens with clumping factor–negative strains, are less specific in their opsonic requirements and less resistant to phagocytosis. In contrast, the mouse-virulent, clumping factor–negative strains behave like encapsulated bacteria. They appear to possess additional surface antigens which delay phagocytosis and

render them more specific in their opsonic requirements. This surface structure may cover antigens ordinarily responsible for clumping. The observation that all of these strains are non-typable raises the possibility that such a surface structure may also block receptor sites required for phage attachment.

Most staphylococci isolated from human infections do not share these characteristics. What then, is the significance of these strains in considering the problem of virulence factors among strains of staphylococci producing

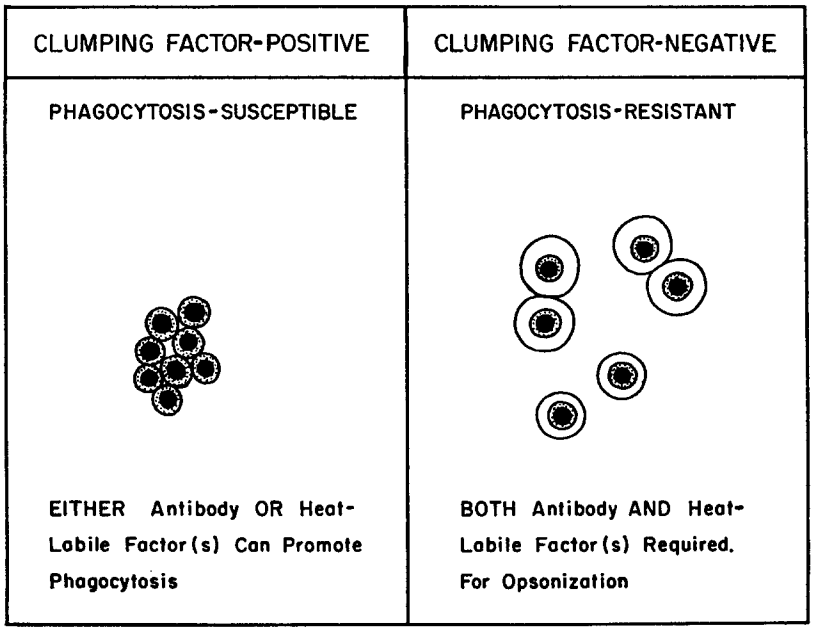


FIG. 4. Pathogenic coagulase-positive staphylococci.

disease in man? It is our current view that these unusual microorganisms may be important biologic indicators of characteristics acquired by pathogenic staphylococci during multiplication *in vivo* which are lost on transfer to laboratory media. Several lines of evidence may be advanced to support this hypothesis.

First, there is tentative evidence which suggests that staphylococci may lose virulence during serial *in vitro* culture. Preliminary work in this laboratory has shown that certain staphylococcal strains obtained from infected lesions are more virulent for mice after but one intervening growth phase than after serial passage on laboratory media. This observation is supported by studies by Van de Velde more than 65 years ago, indicating that the virulence of *Staphylococcus aureus* could be increased by passage through animal tissues (12).

Secondly, it has been shown that virtually all normal adult human sera have detectable opsonizing antibody against the Smith diffuse variant which resembles that evoked in rabbits by immunization (2). This suggests that humans have had wide experience with an antigen similar to or identical with the phagocytosis-retarding Smith antigen. Thus, it seems probable that this antigen is more common than is suggested by studies on most wild type staphylococci.

Thirdly, despite the obvious differences between these strains and staphylococci recently isolated from human infection, there are evidences of close antigenic relationships. While 14 clumping factor–positive strains have not shown resistance to phagocytosis and fail to absorb antibody from Smith diffuse immune rabbit serum, immunization of rabbits with certain of these strains evokes a heat-stable opsonin which promotes phagocytosis of the Smith diffuse variant when heat-labile factors are present (2).

Finally, there are precedents to support the thesis that microbial parasites can acquire important virulence factors during *in vivo* growth which are lost in transfer to laboratory media. For example, certain strains of *Pasteurella pestis* which are indistinguishable from avirulent strains *in vitro* rapidly encapsulate and acquire resistance to phagocytosis during growth within the animal body (13). *Bacillus anthracis* elaborates a lethal toxin when growing in tissues which is not detectable in supernatants of cultures growing *in vitro* (14).

It, therefore, seems reasonable to postulate that these unusual mouse-virulent strains may represent microorganisms which breed true outside the animal host and that other more common forms of staphylococci may acquire similar characteristics during growth in human or animal tissues. Surface antigens retarding phagocytosis appear to be the basic determinants of virulence of these mouse-pathogenic strains in the present experimental system. Studies designed to determine whether such factors can be evoked in other strains growing in tissues are currently in progress.

SUMMARY

Four clumping factor–negative strains of *Staphylococcus aureus* were found to closely resemble the diffuse colonial variant of the Smith strain. All produced fatal intraperitoneal infections in mice, all grew in diffuse, streaming colonies in plasma or serum soft agar, and all behaved like encapsulated microorganisms in *in vitro* opsonic systems.

These staphylococci were resistant to phagocytosis in the peritoneal cavities of normal mice. When mice were immunized with heat-killed vaccines prepared from the Smith diffuse variant these strains were rapidly ingested by peritoneal leukocytes and the animals survived. This observation suggests that these strains share the same or a similar phagocytosis-retarding antigen.

While most pathogenic staphylococci isolated from human material do not behave like these unusual mouse-virulent strains, indirect evidence is cited to support the suggestion that other staphylococci may acquire similar phago-

cytosis-resisting characteristics during *in vivo* multiplication. Studies to support or refute this thesis are in progress.

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