ENHANCEMENT OF *STAPHYLOCOCCUS AUREUS* INFECTIONS IN MICE BY VIABLE SPORES OF *CLOSTRIDIUM TETANI*

by

Clairmont George Drube

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Wayburn S. Jeter
Professor of Microbiology
and Medical Technology

13 January 1967
Date

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ABSTRACT

Staphylococcus aureus injected in a remote area from the site of a previous *Clostridium tetani* spore inoculation resulted in an enhancement of staphylococcal infections without evidence of clinical tetanus. When *Clostridium sporogenes* was used suppression of staphylococcus was noted. Intramuscular and intravenous inoculations of clostridial spores were made in different groups of mice. A comparative study indicated that the intramuscular route for spore injection rendered mice more susceptible to staphylococcal infections when *Clostridium tetani* had been inoculated previously.

INTRODUCTION

Although biologic properties of Staphylococcus aureus may be directly related to its pathogenicity, some experimental studies suggest that changes in host resistance may serve to enhance the infection (1). Alteration of host mechanisms may convert the staphylococcal carrier state into active disease or make a resistant host more susceptible to invasion by potentially pathogenic bacteria.

A large percentage of staphylococcal strains isolated from human infections elaborate an extracellular enzyme, coagulase, which possesses the ability to clot plasma of certain animal species (2), (3), (4). A high correlation exists between coagulase production and virulence of staphylococcal strains. This has led to extensive studies to determine the role of coagulase in establishing infections. These studies were not very convincing as to the direct role of coagulase in staphylococcal infections (5), (6), (1). Many strains of S. aureus produce an extracellular dermal necrotic toxin, which appears inconsequential in initiation of infection (7). Elek found no correlation between the number of staphylococci necessary to produce a cutaneous abscess and the toxin produced in vitro (5). Other extracellular products, such as

leukocidin (7), various hemolysins (8), and hyaluronidase (9), have been found to be elaborated by many staphylococci cultured *in vitro*. The role of these substances in the initiation or maintenance of staphylococcal infections is not clear.

The host responses which may perpetuate staphylococcal infections are intracellular parasitism (6), (10), and the nature of the staphylococcal lesions (11). These may well cause alterations of the host's susceptibility to staphylococcal infection. It is apparent from the relative infrequency of staphylococcal infections, however, that despite the common presence of potentially pathogenic staphylococci within the anterior nares or upon the skin surface (12), there appears to be a high degree of human natural resistance.

Studies reported by Francis indicated that spores of *Clostridium tetani* were stimulated to germination and toxin production *in vivo* in the presence of *S. aureus* (13). Tetanus spores free from toxin are innocuous. Their ultimate fate is either destruction by phagocytes or germination into active vegetative cells which then may elaborate tetanus toxin. Spores injected subcutaneously into mice lie dormant as inert bodies for 3-4 months (14). The anaerobic condition established by *S. aureus* and other

organisms may create conditions that make possible the germination of tetanus spores.

The purpose of this study was to reinvestigate the relationship between S. aureus and C. tetani spores inoculated into mice.

MATERIALS AND METHODS

Animals. Swiss Webster albino mice, from a colony maintained at the University of Arizona in the Department of Microbiology and Medical Technology, were employed. Mice were weaned approximately 25 days after birth. One week after weaning, the mice were selected at random for tests without preference to sex. Their weights varied from 12-16 g. The animals were housed in groups of eight to ten on sawdust in metal cages. Food (Wayne-Lab Block), and water were given ad libitum.

Staphylococcal Bacterial Cultures. Four strains of Staphylococcus aureus were employed as challenge organisms. These were: Strain 209P, a control strain used by United States Food Drug Administration; Zigler strain, isolated from a small child who died from staphylococcal septicemia, University of Iowa Hospital, Iowa City, Iowa; SSCC, a throat isolate from a field clinic of the State Services for Crippled Children in Iowa, and the Wood strain, from the departmental collection. A stock culture of Staphylococcus epidermidis was also used in these experiments. The cultures were maintained on nutrient agar slants.

The medium employed was fresh beef infusion broth. Stock beef broth was prepared by boiling 500 g of lean beef

for 1/2 hour in one liter of distilled water. The broth was cleared by filtration through a double thickness of gauze and brought to the original volume of 1000 ml. The following ingredients were added to one liter of beef broth: 1) 15 g Bacto-peptone; 2) 5 g tryptose; 3) 5 g gelatin, and 4) 5 g of NaCl. The pH of the fresh beef infusion broth was adjusted to 7.5 with 0.1N NaOH. The medium was sterilized by autoclaving for 15 minutes at 121C. Cooling resulted in the formation of a precipitate which was aseptically removed by filtering through a 0.45μ Millipore filter.

Twelve-hour starter broth cultures were prepared from nutrient agar slant cultures of S. aureus. The starter cultures were adjusted to pH 7.5 with 0.1N NaOH and added in 0.5 ml volumes to 4.5 ml of fresh broth medium, incubated for seven hours and used in infection tests.

The number of cocci in broth at the time of inoculation was determined by a Coleman spectrophotometer, Model A. A turbidity of 85 percent light transmission at 605 mμ was employed. Fresh sterile broth was used as diluent. Cultures so standardized gave viable counts of approximately 2×10^7 organisms per ml. In each experiment plates were made in triplicate on nutrient agar from appropriate culture dilutions to ascertain the number of viable organisms. Inoculations were made intra-abdominally and

intramuscularly into the left hind leg and intravenously into one caudal vein of each mouse.

Clostridial Spore Suspension. Washed Clostridium tetani (2583) and Clostridium sporogenes (777) spores were employed for animal inoculation prior to infectivity tests. Cultures were obtained from the departmental collection. Spores were produced on the surface of HA Millipore filter disks underlayed with meat infusion blood agar. The organisms were grown anaerobically in Brewer jars. These were incubated at 37C for one week and then washed from the surface of the filter disk with 0.15M NaCl solution. After four washings in ten volumes of saline with centrifugation at 720 X G, the spores were resuspended in saline and stored at 4C. The spores of C. tetani were freed from toxin by heating for one hour at 80C (14). Viable spore counts of each suspension were made in triplicate on anaerobic blood agar plates. C. tetani spore suspensions yielded approximately 1 X 10^8 per ml, and the C. sporogenes spore suspension yielded about 4 X 10^7 per ml. Constant numbers of viable spores were used throughout the study. Ten thousand viable spores suspended in 0.1 ml saline were injected 7-10 days prior to staphylococcal challenge. Intramuscular injections were made in the right hind leg and intravenous injections were made in a caudal vein.

Observations on Susceptibility. Four observations were made: 1) survival time following injection of S. aureus; 2) gross characteristics of the lesions; 3) cultures from liver, spleen, kidney and heart blood of staphylococcus infected mice, and 4) recovery of viable clostridial spores at time of death or in the event of no death at the end of a 21-day observation period. The clostridial cultures were made from the site of inoculation and the spleen.

EXPERIMENTAL RESULTS

The first experiments were designed to determine whether inoculation of mice with clostridial spores by the intramuscular route influenced later infection with S. aureus.

Quantities ranging from 100 to 10,000 washed spores of C. tetani and C. sporogenes were inoculated into separate groups of mice. Inoculations were made into the right hind legs and the animals were observed for a period of one week. No gross sign of infection was noted.

After the one week initial observation period, four animals of each spore dilution group were inoculated intramuscularly into the left hind leg with a dose of 2×10^6 S. aureus organisms. Controls included four animals that received each spore dilution without subsequent injection of staphylococci and four mice without clostridial spores but injected with staphylococci.

Two S. aureus strains caused minor illness in the tetanus test mice at 2×10^6 dilution of staphylococci. These were the Wood and 209P organisms. The controls were all negative. All mice injected with the tetanus spores developed stiff left hind legs 48 hours after the injection. This was followed by the formation of discrete nodules 5-8 mm in diameter. The nodules were fully

developed in the left inguinal region during the first two weeks after inoculation, but receded during the third week. Three weeks after the staphylococcal inoculation the mice were killed and their tissues cultured. The cultures from all mice were negative.

Animals infected with the Zigler strain, developed stiff left hind legs in both the tetanus spore and staphylococcal control groups. These signs were first observed 48 hours after inoculation, but subsided within 96 hours in the controls. In the tetanus inoculated mice two of the 12 animals developed clubbed left hind feet and five developed large abscesses, 1-2 cm in diameter, in left groin. The five remaining mice recovered by the end of the testing period. Staphylococci were recovered from the left hind legs and spleens of all the mice injected with C. tetani spores and staphylococcal control mice (Table I).

The strongest indication of enhancement of staphylococcal infection was with the SSCC strain. In addition to stiff legs and discrete nodules, the tetanus inoculated groups developed large abscesses in the left groins with areas of induration extending across the abdominal walls. Three of the 12 mice had an enlarged left hind foot. One mouse died on the 11th day. The remaining 11 mice injected with C. tetani spores never regained their normal activity.

TABLE I

RELATIVE PATHOGENICITY OF STRAINS OF S. AUREUS[1]
INOCULATED INTRAMUSCULARLY INTO MICE
PREVIOUSLY INJECTED WITH
CLOSTRIDIAL SPORES[2]

Groups of mice	Strains of S. aureus			
	SSCC	Zigler	Wood	209P
C. tetani spore	4+ *	3+	1+	1+
C. sporogenes spore	-	-	-	-
Staphylococcal control	3+	2+	-	-

\- Negative

1+ Early signs - negative cultures for S. aureus

2+ Early signs - positive cultures for S. aureus

3+ Lesions - positive cultures for S. aureus

4+ Deaths with lesions - positive cultures for S. aureus

* Osteomyelitis

1/2,000,000 organism were injected per animal
2/Clostridial spores were injected intramuscularly

The staphylococcal control mice developed small discrete nodules 0.5-1 cm in diameter at the site of staphylococcal inoculation.

Necropsies showed gross evidence of osteomyelitis in the bones of the left hind legs of the tetanus injected mice. The abscesses contained macroscopic spicules of bone from the immediate areas. Cultures for S. aureus from these abscesses, spleens, left hind legs and heart blood were positive. S. aureus was isolated from the spleens and left hind legs of the control mice but were not recovered from heart blood.

All the mice inoculated with C. sporogenes spores showed no signs of staphylococcal infections.

This first experiment demonstrated that the mouse's natural resistance to staphylococcal infection induced by the intramuscular route was altered by a preparative inoculation of spores of C. tetani intramuscularly. Although the C. sporogenes spores were used for control purposes, they produced a result different from staphylococcal controls and tetanus spore animals in that the mice inoculated previously with C. sporogenes spores were resistant to the doses of staphylococci injected intramuscularly (Table I).

A second experiment was designed to determine whether mice inoculated with staphylococci by a different route would respond in a similar manner. Strain 209P was omitted because of its similarity to the Wood strain. All

experimental mice received 10,000 washed spores, since no apparent differences were noted in the first experiment among the animals receiving different spore dosages. The same type of controls were used as in the first experiment; a clostridial spore control without staphylococcus; a staphylococcal control without clostridial spores, and a nonpathogenic staphylococcal control with clostridial spores. The intravenous and intra-abdominal routes were employed with the same number of staphylococci (2,000,000 organisms per 0.1 ml).

The intravenously infected mice developed lesions in the kidneys and subcutaneous abscesses with involvement of inguinal, iliac and axillary lymph nodes. These lesions are illustrated in Fig. 1.

Although this experiment was not primarily designed to analyze the mechanisms of pathogenicity of staphylococci, it did confirm the fact that intravenous injections of virulent staphylococci in mice commonly results in a fatal outcome due to the rapid and progressive development of abscesses in the kidney (15), (1).

The mice infected intra-abdominally did not usually develop lesions in the kidney, but there was involvement of the mesenteric, iliac, inguinal, cervical and axillary lymph nodes. Some of the animals developed swollen edematous joints in the feet (Fig. 2).

Fig. 1. Gross lesions in mice injected with clostridial spores infected intravenously with S. aureus. Abscesses in kidneys, enlargement of spleens, subcutaneous abscesses and involvement of the axillary, inguinal and iliac lymph nodes.

Fig. 2. Gross lesions in mice injected with clostridial spores infected intra-abdominally with S. aureus. Enlarged spleen, edematous swollen left hind foot and involvement of the cervical, axillary, iliac and inguinal lymph nodes.

The mice injected intra-abdominally and intravenously with the Wood strain of S. aureus did not show gross signs of infection during the three week period, nor were gross lesions observed at necropsy. Cultures from the livers, spleens and kidneys of the intravenously infected animals inoculated with C. tetani spores were all positive for S. aureus. The heart blood from these animals was negative for staphylococci. From the mice infected intra-abdominally with staphylococci and prepared with tetanus spores, S. aureus was cultured only from the spleens. The livers, kidneys and heart blood were negative.

There was no gross pathology observed in the mice inoculated intra-abdominally with the Zigler strain of S. aureus. The only animals in which staphylococci were recovered were the staphylococcal control mice. In these, the organisms were isolated from the kidneys. In staphylococcal control mice injected intravenously, bacteria were also isolated from the kidneys. However, the intravenously infected mice injected with tetanus spores all developed signs of infection, with one death after ten days. All animals developed the characteristic lesions (Fig. 1). The heart blood, spleens, livers and kidneys yielded positive cultures for S. aureus (Table II).

The number of SSCC organisms employed was found to be overwhelming in the intravenously inoculated mice. The staphylococcal control mice all died within 14 days. The

TABLE II

RELATIVE PATHOGENICITY OF VARIOUS STRAINS OF *S. AUREUS*[1] INOCULATED INTRA-ABDOMINALLY AND INTRAVENOUSLY INTO MICE PREVIOUSLY INJECTED WITH CLOSTRIDIAL SPORES[2]

Groups of mice	STRAINS OF *S. AUREUS*					
	Intra-abdominally			Intravenously		
	SSCC	Zigler	Wood	SSCC	Zigler	Wood
C. tetani spore	4+	-	2+	4+	4+	2+
C. sporogenes spore	-	-	-	4+	-	-
Staphylococcal control	1+	1+	-	4+	2+	-

\- Negative

1+ Early signs - negative cultures for *S. aureus*

2+ Early signs - positive cultures for *S. aureus*

4+ Deaths with lesions - positive cultures for *S. aureus*

[1]/2,000,000 organisms were injected per animal
[2]/Clostridial spores were injected intramuscularly

staphylococci at this dose were also lethal for the mice injected with C. tetani and C. sporogenes spores.

The mice injected with spores of C. sporogenes intramuscularly showed no signs of staphylococcal infection by the intra-abdominal and intravenous routes with the Zigler and Wood strains. The dose of the SSCC strain of S. aureus by the intravenous route did elicit demonstrable staphylococcal infections in the mice inoculated with C. sporogenes spores, but these mice did not show any signs of staphylococcal infection by the intra-abdominal route (Table II).

Intravenous inoculations of the SSCC strain were made with 20% of 1-LD_{50} dose of staphylococci. This dose was chosen to represent approximately an LD_{10} (370,000 organisms). The LD_{50} dose was calculated by the Reed-Muench method (16). An additional group of mice was given a spore inoculation by the intravenous route. Previously, mice were inoculated with spores by the intramuscular route. The experiment would demonstrate whether spores inoculated into the blood stream would cause the same effect.

The only mice which showed results parallel to those of the previous studies were the intramuscularly prepared mice. Mice inoculated by the intravenous route with clostridial spores were all negative for infections and isolates of S. aureus. The organism was recovered from each mouse in the staphylococcal control group. All of the

intravenously challenged mice injected with C. tetani spores intramuscularly expired by the 13th day. The gross pathology was similar to that presented in Fig. 1.

The use of fewer staphylococci of the SSCC strain inoculated in the mice intravenously and with clostridial spores injected intramuscularly and intravenously, demonstrated that C. tetani spores by the intramuscular route enhanced staphylococcal infections and the C. sporogenes spores by the same route inhibited staphylococcal infections (Table III).

Table IV is a composite chart of the relative reactions in mice between the various strains of S. aureus and clostridial spores. The portals of entry for the staphylococcus show little if any effect on the enhancement by the C. tetani spores. Inoculations of the Zigler strain by the intra-abdominal route gave results which indicated slightly lessened virulence of this organism by this portal of entry. Although C. sporogenes spores were employed as nonpathogenic clostridium control, they showed an inhibitory effect on staphylococcal infections in all mice. None of the mice inoculated with C. sporogenes spores showed signs of infection and all S. aureus cultures were negative.

Spleens from animals inoculated with clostridial spores were cultured. In each case these cultures yielded the homologous organism for which the spores were

TABLE III

SUSCEPTIBILITY OF MICE INOCULATED INTRAMUSCULARLY AND INTRAVENOUSLY WITH CLOSTRIDIAL SPORES TO ONE LD_{10} DOSE OF S. AUREUS (SSCC) INOCULATED INTRAVENOUSLY[1]

Groups of mice	Routes Clostridial Spores	
	Intramuscular	Intravenous
C. tetani spore	4+	-
C. sporogenes spore	-	-
Staphylococcal control	2+	1+

\- Negative

1+ Early signs - negative cultures for S. aureus

2+ Early signs - positive cultures for S. aureus

4+ Deaths with lesions - positive cultures for S. aureus

[1] $1/LD_{10}$ dose equals approximately 370,000 organisms

TABLE IV

RELATIVE REACTIONS BETWEEN VARIOUS STRAINS OF *S. AUREUS* IN MICE WITH PREVIOUSLY INJECTED SPORES OF *C. TETANI* AND *C. SPOROGENES*[1]

Groups of mice	Strains of *S. aureus*								
	SSCC			Zigler			Wood		
	Routes			Routes			Routes		
	IM	IP	IV	IM	IP	IV	IM	IP	IV
C. tetani spore	* 4+	4+	4+	* 3+	-	4+	2+	2+	2+
C. sporogenes spore	-	-	-	-	-	-	-	-	-
Staphylococcal control	3+	1+	2+	2+	1+	2+	-	-	-

\- Negative

1+ Early signs - negative cultures for *S. aureus*

2+ Early signs - positive cultures for *S. aureus*

3+ Lesions - positive cultures for *S. aureus*

4+ Deaths with lesions - positive cultures for *S. aureus*

* Osteomyelitis

1/ Clostridial spores were injected intramuscularly

inoculated. Muscles from intramuscularly prepared mice were cultured and all were positive for clostridia.

Mice infected with S. epidermidis showed no signs throughout the experiments. Attempts to recover the organism failed in all cases.

DISCUSSION

A report by Francis indicated that when S. aureus and tetanus spores were inoculated in vivo at a single site, such as in muscle, there was a symbiotic effect, with tetanus resulting (13). S. aureus established an area of anaerobiosis which supported germination of the clostridial spores. The vegetative organism of C. tetani, in turn, produced tetanus toxin. In the present study, however, when cocci were injected in a remote area in sufficient numbers there was an enhancing effect on staphylococcal infections without evidence of clinical tetanus. The mechanism of this effect is unknown.

Smith and Dubos studied the susceptibility of mice to systemic staphylococcal infections under varying conditions. Their experiments showed that host resistance was swiftly and abruptly depressed by stress situations as varied as transient starvation or the administration of thyroid or dinitrophenol (1), (17), (18). Observations by Dubos and Schaedler further indicated that such heightened susceptibility may be transient and reversible (19). Our results indicated a different type of stress.

When the host resistance of mice tested was altered, increased susceptibility to staphylococcal infection was found with C. tetani spores and increased

resistance to staphylococcal infection was shown with C. sporogenes spores. The discrepancy between the two groups is not yet explained. The major notable difference is the toxigenicity of C. tetani and the lack of toxigenicity of C. sporogenes. There is no evidence, however, that toxigenicity plays a role in these studies.

SUMMARY

The natural resistance of mice to staphylococcal infections was altered.

Inoculation of mice with C. tetani spores by the intramuscular route enhanced later infections with S. aureus by the intramuscular, intra-abdominal and intravenous routes.

C. sporogenes spores injected intramuscularly increased the resistance of mice to intramuscular, intra-abdominal and intravenous staphylococcal infections.

The effect of clostridial spores on host resistance to staphylococcal infections was greatest when the spores were injected intramuscularly.

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