Serologic Studies of Staphylococcal Enterotoxin

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THE NEED for a simple and specific technique for demonstrating and assaying staphylococcal enterotoxin has been recognized for a long time. Research efforts ranging from biological tests with small and relatively cheap animals (*1*, *2*) to extensive chemical and serologic studies of enterotoxin (*3*) have not attained this important objective.

The many contradictory reports found in the literature (*4*) on staphylococcal enterotoxin are evidence of the inadequacy of the crude, difficult, and impractical tests that are available for its detection. Evidence incriminating suspected foods in outbreaks of staphylococcal food poisoning is largely circumstantial and is limited to the use of epidemiological findings and the demonstration of the presence in the suspected food of appreciable numbers of enterotoxin-producing staphylococci. The very ubiquity of the staphylococcus and, conversely, the possibility of the presence of the heat-resistant enterotoxin in foods which no longer contain viable staphylococci, detract considerably from the value of such procedures.

Furthermore, the demonstration of enterotoxigenicity of the isolated staphylococcus involves considerable effort. The isolated organism must be cultured on special media in order to produce the enterotoxin, and the presence of the latter is determined by the feeding of monkeys or the parenteral introduction of the culture filtrates into monkeys or cats. When available, human volunteers may be fed the suspected food or the culture filtrate. Monkeys, cats, and humans vary considerably in their susceptibility to the enterotoxin and may acquire an increased tolerance to it. Prior to parenteral administration of the culture filtrate it is necessary to remove or neutralize the alpha or beta hemolysins which may be present. These toxins are lethal and may in themselves elicit the emetic reaction characteristic of the enterotoxin. The monkey-feeding test, although specific, is impractical because of its low sensitivity, the marked variation in susceptibility of the animals, and obvious problems of their cost, availability, handling, and maintenance.

Parenteral administration of the enterotoxin to cats is complicated by the activity of the alpha and beta hemolysins, by rapid production of increased tolerance to the enterotoxin, and by a considerable variation in susceptibility of test animals. This method is, however, more sensitive, cheaper, and more convenient than the monkey-feeding test. In the studies presented here, 3 to 10 ml. of culture filtrate was injected intravenously into unanesthetized adult cats, as described by Hammon (*5*). Prior to injection, alpha and beta hemolysins were removed by boiling, neutralization with antiserum containing antibodies for alpha and beta hemolysins, or by digestion with pancreatin (unpublished data). The cats were used once only.

Key strains of staphylococci, selected for the production of enterotoxin for immunization purposes, were checked for enterotoxigenicity by feeding culture filtrates to monkeys.

The studies described here were designed to

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determine the possible value of serologic procedures for the development of a practical test for staphylococcal enterotoxin and were carried out in the following sequence: (*a*) production of enterotoxin in a simply prepared and dialyzable fluid medium; (*b*) separation and concentration of the enterotoxin for purposes of immunization and serologic testing; (*c*) demonstration of antigenicity by the passive transfer of immunity and by in vitro neutralization of enterotoxin, using serum from immunized rabbits; and (*d*) demonstration of an antigen common to enterotoxin-containing culture filtrates and absent in culture filtrates known not to contain enterotoxin.

Production of Enterotoxin

Since staphylococcal enterotoxin is produced in relatively small amounts, it was felt essential that a culture medium be used from which the products of bacterial growth could be easily separated. To this end, a medium was sought which was dialyzable, free from lipids, proteins, and carbohydrates, and capable of supporting good staphylococcal growth and toxin production.

Alpha hemolysin rather than enterotoxin productivity was studied in the early development of the medium, and later the adequacy of the medium for the production of enterotoxin was determined by comparison with preparations made in Dolman and Wilson's soft agar medium (*6*). Aeration by rocking (*7*) or rotation (*8*) of shallow fluid cultures in an atmosphere of 40 percent carbon dioxide and 60 percent oxygen was employed in this portion of the study.

Favorite and Hammon (*8*) suggested the use of a simple medium for the production of enterotoxin, consisting of a hydrolysate of casein enriched with glucose, nicotinic acid, and thiamine. To obtain good growth and toxin production, they found it necessary to inoculate the medium rather heavily with a culture prepared in a meat infusion peptone broth, thus supplying additional growth factors but at the same time introducing complex organic substances. In our hands this medium and procedure failed to give consistently good yields of either alpha hemolysin or enterotoxin.

When a commercially prepared acid hydrolysate of casein (Difco's Casamino acids) was employed, it was found that in order to obtain very good growth of staphylococci it was necessary to add calcium pantothenate, l-cystine, tryptophane, and magnesium sulfate in addition to glucose, nicotinic acid, and thiamine. To obtain good production of alpha hemolysin, it was found necessary to substitute sodium acetate for the glucose and to add an optimal amount of iron. The following formula, which was found to be best for growth and the production of alpha hemolysin, consistently supported the production of enterotoxin.

	Gram
Ferric citrate	0.025
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.2
l-cystine	0.025
Sodium acetate	7.0
l-tryptophane	0.075
Casamino acids (Difco)	20.0
	Microgram
Calcium pantothenate	500
Thiamine hydrochloride	40
Nicotinic acid	1,200
	Liter
Distilled water	1

The pH was adjusted to 7.2–7.4 by the addition of approximately 1.0 ml. of 10 N sodium hydroxide, and the medium was sterilized by autoclaving at 121° C. for 15 minutes after distribution in the final container. Inoculums were small (0.1 ml. of an 18- to 24-hour broth culture) for 30- to 100-ml. quantities of medium and no special atmosphere was required. The method of aeration varied with the volume of toxin desired. The rotation at 20 rpm of 30-ml. quantities of the culture in 8-oz. nursing bottles (*8*), the rocking (*7*) of 100-ml. quantities in Roux bottles through an arc of 20° each 1½ seconds, and the sparging of air through larger volumes (*9*) all proved satisfactory. While enterotoxin was produced in all three procedures after 24 hours, rotated or rocked cultures were harvested after 30 to 48 hours of incubation at 35°–37° C. Larger volumes of culture were aerated by sparging with filtered air for 20 to 24 hours after inoculating with approximately one-tenth volume of a culture in

the same medium which had been incubated for 20 to 24 hours with rocking or rotation.

Separation and Concentration

It was necessary to concentrate and partially purify the crude toxin before use in attempts to immunize rabbits and in the serologic studies described below. This was accomplished briefly as follows: After removal of the organisms by centrifugation and filtration through a Selas candle, the filtrate was reduced in volume approximately 100 times by dialysis and concentration. Dialysis was carried out for 2 or 3 days at 5° C. against distilled water containing 1:1,000,000 merthiolate. Concentration was then accomplished by perevaporation in a current of air or by the use of a flash evaporator.

Some of the larger volumes of culture filtrate were partially purified and concentrated by first adjusting the pH to 3.2–3.3 at 0° C. with 1:5 HCl to precipitate a considerable amount of nonenterotoxic material and then precipitating most of the enterotoxin from the supernatant by slowly adding methanol to a final concentration of 25 percent while maintaining the temperature at −5° C. or lower.

In some of the immunization studies in which toxins prepared with enterotoxic staphylococcus strains No. 224 and No. 230 were used, digestion with crystalline trypsin was found to remove the hemolysins without appreciable destruction of the enterotoxin. After such treatment, however, it was necessary to increase the concentrations of methanol to 40 to 65 percent in order to precipitate the enterotoxin.

The crude concentrates obtained by these procedures varied considerably in potency, containing from 200 to 1,000 cat-vomiting doses per milliliter. A cat-vomiting dose is the minimal amount of enterotoxin which consistently produces emesis in cats. This is determined by injecting intravenously twofold serial dilutions into healthy cats.

The availability, later, of enterotoxins of higher potencies permitted a considerable acceleration in the progress of the present study. These were concentrates of toxins of strains 196E and S6 obtained through the courtesy of Dr. G. M. Dack. The toxins had been produced in a protolysate medium (*10*) and the dry S6 preparation had been considerably purified, using procedures described by Bergdoll (*3*). The crude 196E concentrates contained appreciable amounts of alpha and beta hemolysins and varied in potency, having a dry weight of from 10 to 90 μg. per cat-vomiting dose. The partially purified S6 preparation contained a small amount of alpha hemolysin and no beta hemolysin and consistently produced emesis in cats with as little as 2 μg. dry weight.

Demonstration of Antigenicity

In the exploratory stages of this phase of the study, immunization of rabbits was carried out over a long period of time, using as antigen trypsin-digested concentrates of No. 224 enterotoxin prepared in a variety of ways and injected at variable dosages both intravenously and subcutaneously. Rabbits were found to possess a marked though variable susceptibility to enterotoxin. When the intravenous route of injection was employed, some rabbits were killed in 12 to 24 hours by 1 or 2 cat-vomiting doses. It was necessary, therefore, to build up resistance to the enterotoxin gradually. One or two cat-vomiting doses in 1 or 2 percent alum were repeatedly injected intracutaneously or subcutaneously, or both, until a tolerance to the intravenous injection of approximately two vomiting doses of the enterotoxin was established. The amount of antigen was then increased and injected subcutaneously. The intervals between injections were adjusted according to the animal's maintenance of weight. In later stages of the immunization, in which larger amounts of antigen were used, the alum content was increased to 10 percent and the antigen was administered subcutaneously in divided doses. All alum-containing antigens were adjusted to a final pH of 6.0 and were preserved with 1:10,000 merthiolate.

Antigens prepared with 196E concentrates or with the partially purified S6 enterotoxin were not subjected to tryptic digestion. Both potassium alum and Freund's adjuvants (emulsion of 2 parts of antigen dissolved or suspended in saline, 1 part Falba, and 1 part paraffin oil, with 0.02 percent killed human

tubercle bacilli and preserved with 1:10,000 merthiolate) were used in the preparation of 196E antiserums (*11*). For the preparation of anti-S6 serums, only Freund's adjuvants were employed.

Serums were obtained from the rabbits under immunization from time to time and tested for their antienterotoxin content either by preliminary mixture with approximately 2 vomiting doses of homologous enterotoxin (neutralization) 1 or 2 hours before injecting cats or by their ability to produce in cats a passive immunity (protection) to approximately 2 vomiting doses of enterotoxin. In the latter procedure the intravenous injection of the serum was followed within 3 to 5 minutes by injection of the challenging dose of enterotoxin. The amount of serum required to demonstrate neutralization or protection varied with the rabbit source, the duration of the immunization procedure, and the potency and adjuvant composition of the enterotoxin antigen.

With the more potent enterotoxins 196E and S6 it was possible to demonstrate the production of "protective" antienterotoxin in a relatively short period of time after tolerance to the enterotoxin had been produced by the repeated intracutaneous, subcutaneous, and intravenous injection of 1 or 2 vomiting doses. For example, a rabbit prepared in this fashion could be injected subcutaneously with 1,000, 2,000, and 3,000 vomiting doses of 196E in 10 percent alum at 14-day intervals. Protection against 2 vomiting doses of the homologous enterotoxin was demonstrated with 0.3 ml. of serum obtained 10 days after the final injection of the antigen. Another similarly prepared rabbit was immunized with 25, 125, 250, and 375 vomiting doses of S6 antigen containing Freund's adjuvants. Protection against two vomiting doses of the homologous enterotoxin required the use of 0.8 ml. of serum obtained 30 days after the final injection of antigen. As little as 0.08 ml. of an anti-196E serum was found to confer a passive immunity against its homologous enterotoxin. In most instances, however, 0.3 to 0.5 ml. of serum was required to produce this effect.

The results of more than 65 tests, representing repeatedly confirmed observations, are summarized in the table. Results obtained with serum fractions prepared according to the procedures of Nichol and Deutsch (*12*) and Aladjem and Lieberman (*13*) are included. Quantitative determinations of the antienterotoxin content of the antiserums were not made. In no instance, however, was more than 0.5 ml. of the serum fraction, or its equivalent of whole serum, used. The antigenicity of the enterotoxins from the three staphylococcus strains, Nos. 224, 196E, and S6, is clearly demonstrated. All react with their homologous enterotoxins, and 196E antiserum protects against heterologous S6 and 230 enterotoxins. Pseudoglobulin as well as the globulin fractions of Nichol and Deutsch prepared from 196E antiserums neutralized 196E enterotoxin and protected cats challenged with 196E and 230 enterotoxins.

Demonstration of a Common Antigen

With the production of serums containing antibodies for homologous and for heterologous enterotoxins, an attempt was made to reveal a serologic relationship between enterotoxin from different strains of staphylococcus. It was felt that the establishment of the serologic identity of staphylococcal enterotoxin or the pres-

Neutralization of enterotoxins by rabbit antiserums and passive transfer of immunity as determined by the intravenous test on cats

Antiserum		Challenging enterotoxin	Results	
Immunizing antigen	Modification of serum		Neutralization	Protection
224	None	224	+	
224	do	230	−	
196E	do	196E	+	+
196E	Pseudoglobulin [1]	196E	+	+
196E	None	230		+
196E	B, C-1, C-2 fractions [2]	230		+
196E	Pseudoglobulin [1]	230		+
196E	None	S6		+
S6	do	S6		+

[1] Pseudoglobulin prepared according to procedure of Aladjem and Lieberman (*13*).

[2] Fractions prepared according to procedure of Nichol and Deutsch (*12*).

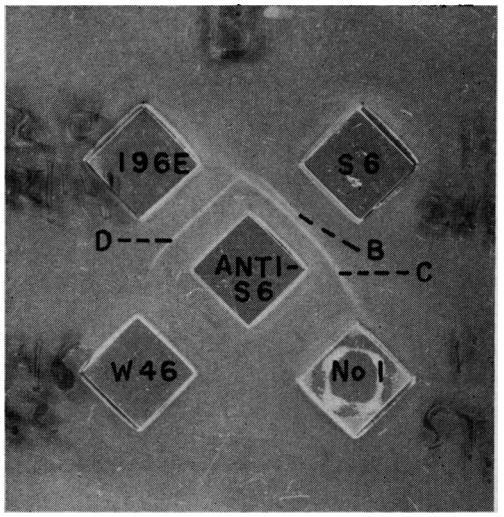

Figure 1. Zones of precipitation after absorption of anti-S6 serum with Wood 46 and No. 1 concentrates and an extract of No. 243 organisms. Illustrating the formation of a "line of identity" (the coalescence of lines D and B) and a "line of nonidentity" (the intersection of lines D and C). Evident also is the antigen excess effect obtained with the concentrated culture filtrate of strain No. 1 which, in addition to failing to produce a zone of precipitation, prevents the complete development of line B.

ence of a distinguishing antigen common to enterotoxic strains might permit the development of a test for the detection and assay of this toxin. A slight modification of the Petri-dish agar diffusion method of Ouchterlony (*14*, *15*) for the qualitative analysis of soluble antigens and antibodies was used for this purpose.

In Ouchterlony's method, antiserum is added to a centrally located well in the agar and antigens are added to peripheral wells. Antigens and antibodies diffuse toward each other through the agar to form zones or lines of precipitation where the two combine in optimal proportions. The method permits separation of multiple precipitation systems into their individual components and, in addition, permits the comparison of two antigens or antibodies with each other in order to establish their identity, partial identity, or nonidentity (fig. 1).

In our study, plates were prepared with a base containing 1.6 percent Noble Special Agar (Difco), 0.8 percent NaCl, 0.01 percent merthiolate, 0.003 percent methyl orange, and M/25 veronal. The mixture was melted, adjusted to pH 7.4 and filtered through paper until clear. A central well and four peripheral wells were prepared using rectangular aluminum blocks, measuring 8 mm. square in cross section. A central hole, measuring 2 mm. in diameter was bored through the length of each block to facilitate its removal from the solidified agar. The wells were prepared by covering the bottom of a standard Petri dish with 10 ml. of the agar. After solidification, the blocks were placed in position and an additional 20 ml. of the agar was added and allowed to solidify. The blocks were carefully removed from the agar and arranged as recommended by Wilson and Pringle (*16*), with a distance of 7 mm. between the central serum-containing well and the peripheral antigen-containing wells. The plates were allowed to remain uncovered for 30 to 60 minutes at 35° C. prior to use. The wells were charged with 0.2 ml. quantities of the reagents and were not refilled. Results were recorded after 5 to 8 days at room temperature.

The S6 antiserum was found to contain antibodies for antigens possessed by both enterotoxic strains and nonenterotoxic strains of staphylococcus. It appeared necessary, therefore, to remove most of the antibodies for these common antigens before attempting to demonstrate the presence of an antigen peculiar to enterotoxic preparations and absent from those that were enterotoxin free.

To attain this goal, anti-S6 serum was first absorbed with concentrated filtrates of enterotoxin-negative strains Wood 46 and No. 1. Both strains gave negative tests for enterotoxin when injected into cats in amounts equivalent to 50–60 ml. of crude culture filtrate. The Wood 46 strain was obtained from the Connaught Laboratories, Toronto, Canada, in 1938. It is coagulase-positive, produces an appreciable amount of alpha hemolysin, and has also been found to be nonenterotoxigenic by other investigators. Strain No. 1 was isolated in 1949 from the stomach contents of an infant who succumbed to what appeared to be staphylococcal food poisoning. This strain differed from nonenterotoxigenic strains and resembled enterotoxin-producing strains in that it contained heat-stable antigen which was precipitated by anti-

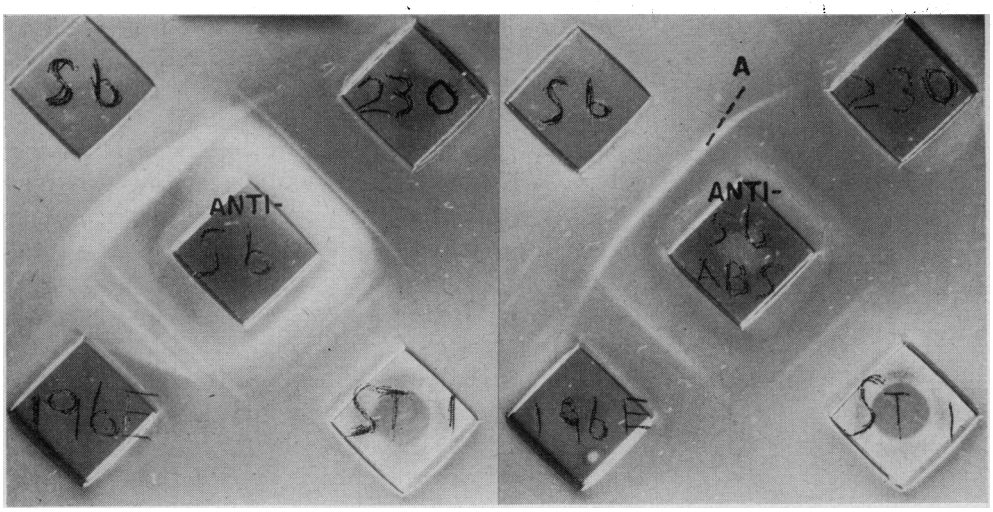

Figure 2. Effect of absorption of anti-S6 serum with concentrates of culture filtrates of strains Wood 46 and No. 1. Many of the precipitation systems produced with unabsorbed serum (left) are not obtained with the absorbed serum (right). Detection of coalescence of lines produced by heterologous 196E and No. 230 enterotoxins with one produced by the S6 antigen-antibody system is interfered with by heavy line A.

enterotoxic serum absorbed with a concentrated culture filtrate of the Wood 46 strain.

The effectiveness of the absorption procedures was determined by preparing Ouchterlony plates to demonstrate coalescing lines of precipitation (lines of identity) between S6 and 196E (see fig. 1) enterotoxins and the absence of such lines in the antigens used for absorption. For this purpose, the peripheral wells were charged with 100-fold concentrates of culture filtrates of the Wood 46 and No. 1 staphylococcus strains and with 2 to 4 cat-vomiting doses of S6 and 196E enterotoxins. Undiluted absorbed serum was placed in the central well.

Although absorption with the Wood 46 and No. 1 antigens removed much of the antibody in the serum (fig. 2), demonstration of a "line of identity" belonging only to the S6 and 196E toxins was not possible. This was due (*a*) to the possession by the No. 1 strain of staphylococcus of an antigen which was common to the enterotoxigenic strains although not found in the other nonenterotoxigenic strains under study, and (*b*) to the presence in the anti-S6 serum of an antibody which was not sufficiently absorbed by the Wood 46 and No. 1 concentrates so that a heavy masking zone of precipitation was produced in the Ouchterlony plate with S6 enterotoxin (fig. 2, right).

Culture filtrates of enterotoxigenic strain No. 243 differed from others under study in that, like the S6 enterotoxin, they produced a heavy masking zone of precipitation with the absorbed anti-S6 serum. Further absorption of the serum with a glass bead extract (*17*) of the cells of strain No. 243 removed the masking antibody. The cell extract rather than a concentrated culture filtrate was employed because of the presence of a smaller amount of enterotoxin in the cell extract. Assay for enterotoxin by injecting cats and for "interfering antigen" by titration with the Wood 46 strain No. 1-absorbed anti-S6 serum showed that the concentrated culture filtrate contained 7 times more interfering antigen but 30 times more enterotoxin than did the cell extract. Absorption of the absorbed serum with the extract of the No. 243 cells resulted in the production of a good, well-defined line of identity between the undiluted absorbed serum and S6 and 196E enterotoxins. Use of the more toxic concentrate of the culture filtrate of strain 243 instead of the cell extract for this purpose resulted in the production of a markedly less well-developed line of identity (fig. 3).

The effects of absorption with the enterotoxin-negative Wood 46 and No. 1 strains and with the No. 243 preparations are presented in

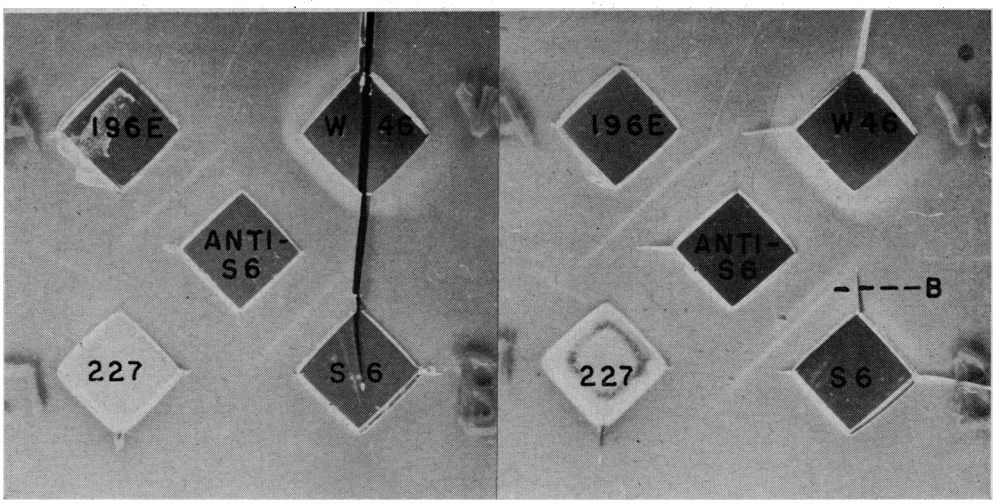

Figure 3. Effect of further absorption with either a concentrated culture filtrate of strain No. 243 (left) or an extract of No. 243 cells (right). Both absorbing antigens remove the heavy line of interference seen in figure 2. Absorption with the cell extract, however, removes less of the antibody which produces the single line with 196E and the more peripheral (line B, right) of the two lines produced with S6 antigen. The nonenterotoxic Wood 46 and No. 227 antigens produce no lines.

figures 2 and 3. The interference line of S6 was removed and only one good line of precipitation was formed with the heterologous 196E toxin. The outer of the two remaining lines formed with the homologous S6 toxin (line B, figs. 1 and 3) could be identified with the single sharply defined line formed with 196E toxin by placing these antigens in adjacent wells to produce clear-cut lines of precipitation which coalesced at the junction of the respective precipitation lines (fig. 1).

The absorbed S6 serum was used finally, to test 100-fold concentrations of culture filtrates of 21 strains of staphylococcus which had been found to be enterotoxigenic by the cat test and 6 enterotoxin-negative strains for their ability to produce "lines of identity" with 196E and S6 toxins. Of the 21 enterotoxigenic strains, 16 were isolated from foods epidemiologically incriminated in food poisoning incidents. Two were isolated during routine examination of foods not involved in food poisonings. Two were isolated from feces from patients with enteritis following intensive antibiotic therapy, and one (No. 244) from the nose of a child.

Typical results obtained with some of the culture filtrates are shown in figure 4. All but one of the enterotoxigenic strains gave lines of identity with both toxins. The one exception, No. 244, was not a food poisoning strain but was isolated from the nose of a child. This strain gave a positive cat test for enterotoxin shortly after isolation, but, when retested, was found to have lost this ability. Of the 6 enterotoxin-negative strains, 5 produced no lines of any kind. The sixth, however, produced a well-defined line of identity with the two positive controls. This strain, No. 260, had been isolated during routine bacteriological examination at the New York City Department of Health Laboratories from crab meat which was not involved in a food poisoning incident.

The antigenic component common to the 21 enterotoxigenic strains is shared by strains No. 260 and No. 1, both of which are apparently nonenterotoxigenic. Strain No. 1 produces this antigen in relatively large amounts. This was shown by an antigen-excess effect when its concentrated culture filtrate was used in the Ouchterlony plate test. It failed to form its portion of the line of identity and also inhibited the development of the portion produced by the enterotoxic control (line B in fig. 1). Furthermore, the antibody in the absorbed serum responsible for the production of the lines of identity was present in small amount and could

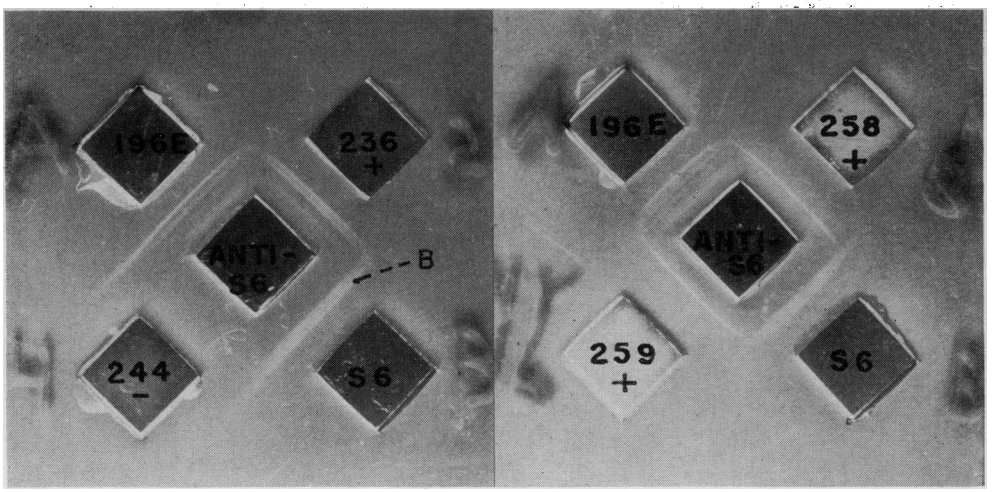

Figure 4. Examples of coalescing zones of precipitation ("lines of identity") formed between enterotoxic (designated with a plus sign) culture filtrates and the two control enterotoxins (S6 and 196E). Left: The line of precipitation produced with enterotoxic culture filtrate of strain No. 236 coalesces with the single 196E line and with the outer (line B) of the two lines produced by the S6 enterotoxin. The nonenterotoxic No. 244 produces no lines.

be removed completely by further absorption with the strain No. 1 concentrate. It was apparent, therefore, that it would be desirable to eliminate the use of this antigen for absorption of the anti-S6 serum.

Use of diluted anti-S6 serum after absorption with Wood 46 cells alone was subsequently found to be a better procedure for the procurement of an absorbed serum with which to demonstrate the antigenic component common to the 21 enterotoxic and 2 nonenterotoxic preparations. Wood 46 organisms removed from air-sparged culture by filtering and centrifuging were suspended in saline to give a heavy suspension, cooled to 4° C., and poured into 15 volumes of acetone at about −20° C. After standing overnight at −15° to −20° C., the acetone was removed and the cells were washed three times by suspending in acetone at −15° C. and centrifuging at −10° C. The organisms were then dried under vacuum and over $CaCl_2$ and stored at 5° C. Absorption of anti-S6 serum with acetone-dried Wood 46 cells was carried out by adding 10 percent (W/V) of the dried organisms to the serum, shaking until a uniform suspension was obtained, incubating at 37° C. with occasional shaking during a period of 2 to 3 hours, and placing at 5° C. overnight. The cells were removed by centrifuging at 20–25° C. and washed with enough saline to adjust the absorbed serum to its original volume. The serum was absorbed a second time in the same manner and examined in order to determine the dilution giving a sharp

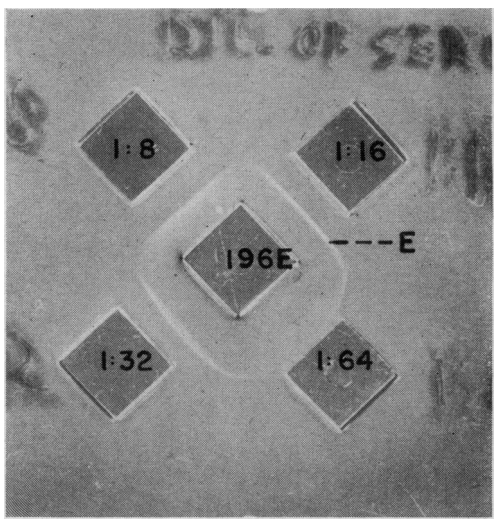

Figure 5. Effect of diluting anti-S6 serum (in peripheral wells) after absorption with Wood 46 cells, on the definition of the single line produced with the heterologous 196E enterotoxin (in central well). Use of a 16-fold dilution of the absorbed serum results in the production of the most sharply defined line (line E).

line with the heterologous 196E enterotoxin in the agar diffusion plate test (fig. 5).

The presence, in strains No. 1 and No. 260, of the antigen otherwise common only to enterotoxigenic strains, suggested the possibility that the use of other culture media and procedures might reveal their enterotoxigenicity. Cultivation of these strains in a mixture of 40 percent carbon dioxide and 60 percent oxygen instead of air and on Dolman's soft agar has yielded negative results. Attempts to demonstrate in the products of growth of these strains a protoxin similar to those described for some of the clostridial toxins (*18–20*) have yielded essentially negative results. In addition, attempts passively to protect cats against S6 enterotoxin with antiserum prepared against concentrated culture filtrates of strain No. 1 have also been unsuccessful, further indicating that the "common antigen" is neither toxin nor protoxin.

Discussion

The work of Dolman and Wilson (*21*) contributed substantially to the direction of the studies presented here. Using antiserum produced in a horse, they were able to demonstrate a specific flocculation reaction which appeared to involve the enterotoxin and its homologous antibody. They indicated the desirability of support of their findings with many flocculation tests performed "in conjunction with parallel kitten tests, and involving filtrates derived from a large number of strains."

The absence of such confirmative studies, in the light of present knowledge, would seem to be attributable to the difficulties involved in the production of a specific antiserum of sufficient potency and the necessary dependence on one or more of the inadequate tests for the enterotoxin. The presence of not more than 5 percent enterotoxin in the purest preparations so far prepared by Bergdoll (*22*) and the consistently positive emetic responses obtained in the present work with 2 μg. of the partially purified S6 enterotoxin suggest a possible explanation for earlier failures to confer passive immunity (*5*, *23*), since in these studies antiserums were produced by immunization with relatively weak and crude preparations. In addition, inability to obtain an active immunity to heterologous enterotoxins in monkeys (*24*) and in cats (*25*) may have discouraged such studies.

Conflicting reports as to the antigenicity of staphylococcal enterotoxin (*5*, *6*, *23*, *26*, *27*) indicated the need for studies designed to resolve this important phase of the problem. Although repeated demonstrations of the production of an acquired immunity or "increased tolerance" to enterotoxin had been made, the evidence for passive transfer of immunity was lacking.

Neutralization of enterotoxic preparations, however, had been reported. Dolman, Wilson, and Cockroft (*28*) found that the serum from a kitten made resistant to enterotoxin neutralized the enterotoxin when a mixture of the two was injected into a normal kitten. Davison, Dack, and Cary (*29*) confirmed this report when the mixtures were injected intraperitoneally. When the intracardial route of injection was used in kittens, or the intravenous route in monkeys, there was no neutralization by the serum, they reported. They also stated: "When normal kitten blood was added to a mixture of enterotoxic filtrate and antiserum and then injected intraperitoneally into normal kittens, protection was not assured." These investigators concluded that the presence of whole blood, extravascularly or intravascularly, prevented the neutralization of enterotoxin by its antiserum. Dolman (*27*), however, demonstrated neutralization of enterotoxin by serum from immunized human and animal sources by injecting enterotoxin-serum mixtures intravenously into cats. Hammon (*5*), working with crude toxins, failed to demonstrate in vivo neutralization through the use of "hyperimmune" serums from cats and rabbits. Surgalla, Bergdoll, and Dack (*30*) were able to demonstrate a neutralization of S6 enterotoxin after mixing the toxin with the homologous rabbit antiserum and feeding the mixture to monkeys. More recently, Burbianka (*31*), administering enterotoxin and antiserum to cats by the intravenous route, claimed to have neutralized a minimal enterotoxic dose of enterotoxin with as little as 0.005 ml. of an antiserum produced in rabbits. Antiserum, produced against enterotoxin derived from one strain of staphylococcus neu-

tralized the enterotoxin produced by other strains.

The evidence obtained with antigens prepared with S6, 196E, and 224 enterotoxins establishes conclusively the antigenicity of staphylococcal enterotoxin. The conferring of a passive immunity to cats injected successively with antiserum and challenging enterotoxin showed effective combination between the toxin and its antibody in the presence of whole blood.

The protection in cats obtained when 196E antiserum was challenged with heterologous 230 and S6 enterotoxins is of special interest in view of the conflicting report of Surgalla, Bergdoll, and Dack (*24*). These investigators, using the monkey-feeding test, found that monkeys possessing an active immunity to 196E enterotoxin were susceptible to S6 enterotoxin. The desirability of further work to demonstrate a passive immunity in the monkey when the challenging enterotoxin is given by mouth suggests itself. The possibility cannot be excluded, however, that differences in avidity or combining power of toxin and antitoxin or in the amounts of available antitoxin may be responsible for the conflicting observations. The short incubation period in staphylococcal food poisoning suggests the need for rapid neutralization of the enterotoxin.

The 21 "positive" culture filtrates appear to be serologically related and set apart from the 6 "negative" culture filtrates when examined by the agar-diffusion Petri dish method of Ouchterlony. The occurrence of the antigen common to the 21 enterotoxigenic strains in preparations obtained from 2 of 8 nonenterotoxigenic strains does not permit, at this time, the use of this antigen for the development of a reliable serologic procedure for the detection of enterotoxigenic strains. Should strains No. 1 and No. 260 eventually be found to be enterotoxigenic, more strains should be studied to establish the reliability of such a test. Furthermore, before the antigen can be identified with enterotoxin, a clear-cut demonstration of its enterotoxicity should be made.

Summary

A completely dialyzable fluid medium for the production of staphylococcal enterotoxin has been developed.

The antigenicity of staphylococcal enterotoxin and in vivo protection of cats against enterotoxin of heterologous and homologous strain origin has been demonstrated.

Of 29 strains of staphylococcus examined for enterotoxigenicity by the intravenous cat test 21 enterotoxigenic and 2 nonenterotoxigenic strains were found to be serologically related and set apart from the remaining 6 nonenterotoxigenic strains when examined by the agar diffusion test of Ouchterlony.

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John K. Hoskins, 1884–1958

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