Staphylococcus Enterotoxin: An Improved Cat Test, Chemical and Immunological Studies

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AN attempt has been made in the studies here reported to evaluate the biological tests recommended for the detection of the presence of staphylococcus enterotoxin and to simplify and standardize the interpretation of the most reliable of these. At present most health department laboratories find it impractical to test for this very common food poisoning substance because of inherent difficulties of the available tests and the almost complete lack of knowledge regarding the chemical nature of the toxin and of how to extract it from suspected foods. When it was felt that the criteria for a reliable, inexpensive, practical and highly specific test had been met, by means of it preliminary studies were made of the physical, chemical, and immunological properties of the toxin.

QUALITATIVE TESTS

Since Dolman, Wilson, and Cockcroft [1] described the intra-abdominal kitten test, other *in vivo* tests have been abandoned to a large degree. Man, although probably the most susceptible of all animals, for obvious reasons is now seldom employed. Monkeys have been extensively used but data have been accumulating which indicate that these animals have relatively low susceptibility, show great variation in their response, and give occasional false positive reactions.[2-5] In our experience monkeys have proved very unsatisfactory and perhaps the greatest single objection lies in the fact that they are not readily available to many laboratories.

Dolman Kitten Test—

The kitten test as described by Dolman and his associates [1] involves the injection of specially prepared culture filtrates into the abdominal cavity of very young kittens (250–500 gm.) after inactivation of the lethal exotoxins by heat, formalin, or neutralization by specific antitoxin. After a variable period of time, toxic filtrates will produce vomiting and diarrhea. These workers indicate that the same kitten may be used repeatedly within limits, and Dolman and Wilson [6] set these limits at 7 to 10 days, explaining that after a period of 2 to 3 weeks a significant degree of immunity may develop. Kupchik,[7] although confirming the usefulness of the test, believes that an animal should be used a second time only after a period of 2 weeks has elapsed. Rigdon [8] and Jones and Lochhead [9] report nonspecific reactions following the inoculation of control materials.

During the early phases of this work

false positive reactions and the rapid resistance gained through previous inoculation presented severe difficulties, but before abandoning the method an experiment was planned to determine if possible the chief factor responsible for the rapidly developed tolerance. It was suspected that peritoneal irritation produced the nonspecific vomiting and diarrhea and, if this could be incriminated as playing a rôle in the development of resistance, the route of inoculation should be abandoned, but not necessarily the animal.

A group of 7 previously unused kittens all weighing about 1,000 gm. were inoculated intra-abdominally at intervals of 2 days with various materials in equal amounts by weight; these were: known enterotoxic filtrate,* filtrate from known nonenterotoxigenic strains of staphylococci * identically prepared, uninoculated sterile infusion broth similar to that used for toxin preparation, and infusion broth containing 0.3 per cent formalin (Table 1). All cats were tested at the end of the experiment (fourth inoculation for most animals) with the enterotoxic filtrate, together with 2 new control animals. Vomiting accompanied by diarrhea occurred 3 times and nausea and diarrhea once from the inoculation of nonenterotoxin containing material. The various control materials given to 5 kittens on repeated inoculation produced almost the same degree of tolerance to the final test dose of enterotoxin as did repeated inoculations of enterotoxin to 2 kittens receiving this material only, and the

TABLE 1

Results of Multiple Intra-abdominal Inoculations of Enterotoxin and Nonenterotoxic Materials in Equal Doses, at Regular Intervals, in Previously Unused Kittens

Days between Inoculations		—		2		2		2		10	
Cat No.	*Weight in Grams*	*Inoculum*	*Reaction*	*Inoculum*	*Reaction*	*Inoculum*	*Reaction*	*Inoculum*	*Reaction*	*Inoculum*	*Reaction*
1	1,000	C.P.[1]	+++	C.P.	0	C.P.	±	C.P.	0	C.P.	0
2	1,050	C.P.	++++	C.P.	+	C.P.	+	C.P.	0	C.P.	+
3	900	Broth	0	Broth	0	Broth	0	C.P.	0	C.P.	+
4	1,400	Formalin broth[2]	0	Formalin broth	+*	Formalin broth	0	C.P.	+	—	—
5	800	W-46[3]	+++	43[4]	±	W-46	0	C.P.	+	—	—
6	1,200	—	—	Autolysate C.P. organisms	0	Formalin broth	+*	C.P.	++	—	—
7	1,250	—	—	W-46	0	43	0	C.P.	++++	—	—
8	1,200	—	—	—	—	—	—	C.P.	+++	—	—
9	1,200	—	—	—	—	—	—	C.P.	++++	—	—

[1] C.P. = Enterotoxigenic strain. [2] Formalin broth = 0.3 per cent formalin in infusion broth.
[3] W-46 = "Wood" strain, nonenterotoxigenic. [4] 43 = Nonenterotoxigenic strain.
* Vomiting occurred less than 15 minutes after inoculation.

degree of tolerance in all varied in direct proportion to the number of previous inoculations, regardless of the materials employed. Three of the animals were again inoculated (fifth inoculation) after a 10 day rest period, and 2 which previously failed to react again responded in a positive manner. We conclude from this that peritoneal irritation resulting from the inoculation

* Staphylococci used in this experiment and others here reported were obtained from the following sources:
W-46, nonenterotoxigenic Wood strain obtained from Dr. Dolman.
43, nonenterotoxigenic strain isolated from the Bundaberg disaster, obtained from Dr. Dack.
C.P.; C.C.P.; W.C.; and C.E. isolated by the author from cream filled pastries sent to this laboratory by the Massachusetts State Department of Public Health. These pastries were incriminated in four separate outbreaks of food poisoning. All four strains are yellow pigmented staphylococci and produce high titers of enterotoxin, with varying amounts of *alpha* and *beta* hemolysins.

of even sterile culture medium results in slower or less complete absorption of subsequently inoculated toxic materials. After a prolonged rest period this low grade peritonitis clears and normal absorption may again occur. It was not possible to determine through this experiment whether or not specific immunity results from repeated inoculations of enterotoxin, for the nonspecific peritonitis masked the other possible effects.

Intravenous Kitten Test—

Davison, Dack, and Cary [10, 11] have used the intravenous and intracardiac routes for inoculating cats. In our experience, which includes over 175 intravenous inoculations of toxic material, this route provides the method of choice. We have been pleased to find that not only were nearly mature or adult cats more easily handled and inoculated, more easily obtained, and less susceptible to epizoötic disease, but they were also more susceptible to toxins inoculated by the intravenous route than are the tiny kittens employed by Dolman and his associates. For all practical purposes the same amount of toxin can be expected to produce a similar response in any animal ranging from about 800 gm. to the largest cat.

To perform the test, the cat, without anesthesia, should be placed on its back on an animal board and the inner aspect of one thigh shaved to a point just below the knee. Slight pressure over the vein near the inguinal region enables one easily to see and enter the saphenous vein at about the level of the knee, or just above, with a sharp 26 gauge needle. After releasing the pressure above, the syringe and leg should be grasped together with one hand and the injection made slowly. Depending on the toxin, from 0.5 ml. to 5 ml. may be required. The toxin must of course be first treated to inactivate the lethal factor or factors. This we find most convenient to do by heating a small amount for 30 minutes in a boiling water bath. The precipitate should be sedimented and the supernate used as inoculum. When heat is used, the supernate from well centrifuged cultures may be safely used without filtration. Sterility, in practice, appears to be unnecessary. So far, although 49 inoculations of many different materials which did not contain enterotoxin have been made by this route, some of them in doses up to 12 ml., no nonspecific vomiting has occurred.* Only one cat has died as a result of inoculation of either toxic or nontoxic materials and this was due to an immediate anaphylactic-like reaction after rapid inoculation of a large dose of enterotoxin in a cat which had received many previous inoculations.

After repeated inoculation tolerance develops, but this occurs to a much less degree than when the intra-abdominal route is used. No single experiment has been planned to demonstrate the degree of resistance developed, but an analysis of the protocols of individual animals is productive of information in this regard. We have never found any cat which failed to vomit on first intravenous inoculation with a reasonable dose of enterotoxin. Great individual differences occur, however, in apparent degree of tolerance acquired and its rapidity of development. A dosage of 2.0 ml. of toxin of average potency will produce a severe reaction on first inoculation. A somewhat less severe reaction will usually occur on repeating this dose, apparently regardless of the time interval following the previous inoculation. An increase of 50 per cent to 100

* Since submitting this paper for publication, aqueous extracts of fish, after dialysis, have produced nausea and vomiting in cats. This work was done at the George Williams Hooper Foundation, University of California, with Mr. C. W. Lang and associates.

per cent in dosage for the third inoculation gives a moderate or severe reaction. At the next a further increase in dosage is frequently required. Because of the uncertainty of response and the increased volume of inoculum necessary, we seldom use an animal the fourth time, and never accept as final the results of a negative test unless repeated on at least 2 previously unused animals in a minimal dose of 3 ml.

The following experiment demonstrates that the acquired tolerance is not lost, at least in entirety, during a period of rest. Six cats (see Table 2) which had been inoculated with enterotoxin from 1 to 10 times previously, were given a complete rest for 2 months, then all were retested with a 3 ml. dose of a potent toxin. The results are similar to those which might have been expected had no rest period been allowed.

TABLE 2

Effect of a 3.0 Ml. Intravenous Test Dose of Enterotoxin on Cats with Varied Experience After a 60 Day Rest Period

Cat	Number Previous Inoculations	Time in Minutes Nausea Noted	Time in Minutes Vomiting Occurred	Degree of Diarrhea
1	1	39	40	++++
2	2	38	40	+
3	2	55	58	+
4	6	63	—	0
5	7	55	—	0
6	10	—	—	0

The train of symptoms observed after an intravenous inoculation of enterotoxin differs little from that described by Dolman, Wilson, and Cockcroft [1] following intra-abdominal inoculation. In from 15 minutes to 2 hours, most frequently in about 30 minutes, vomiting occurs, preceded by nausea. Coarse tremors are usually noted and the hair stands erect. This we interpret as a chill or rigor for we have found that the temperature soon begins to rise, usually to 104° F. or 106° F., attaining this maximum only after 2 to 4 hours. This rise in temperature after a chill also occurs following intra-abdominal inoculations, but we are not aware that it has been previously described. Diarrhea, although it occurs in most animals and is frequently very conspicuous, may occasionally be of a very mild nature. When present it usually persists for several hours. Vomiting may recur at intervals of from 5 minutes to 1 hour over a period of 2 or 3 hours. After 3 to 4 hours from the onset of symptoms, the cat is frequently noticeably less ill and 24 to 48 hours later it appears normal if opportunity has been afforded for rehydration. Not infrequently animals develop diarrhea following inoculation of control materials, so vomiting only can be accepted as specific. Since vomiting and occasionally diarrhea are conspicuous symptoms of a very common and highly infectious type of feline epizoötic, panleucopenia (encountered and studied by Hammon and Enders [12, 13] concurrently and independently with Lawrence and Syverton,[14, 15]) it is quite important that the cats used be known to be in good health. Enders and Hammon [16] have described a satisfactory method of actively or passively immunizing experimental animals. A moderate size meal eaten shortly before the inoculation of enterotoxin has been found to increase the effectiveness of the vomiting stimulus, and noted refusal of the offered meal aids distinctly in the elimination of sick animals.

Use of Chick Embryos—

Very careful tests were repeatedly made to determine whether inoculation of developing chick embryos might serve as a method of detecting the presence of enterotoxin. Inoculations were made onto the surface of the chorio-allantoic membrane through an artificial air space, also directly into the yoke sac, but without any apparent effect upon the life of the embryo.

CHEMICAL AND PHYSICAL STUDIES

Using the intravenous cat test which had been found to be highly specific for culture toxins (after inactivation of other toxic factors and observing the criteria mentioned above, *i.e.*, interpreting only as positive those materials which produced definite vomiting and accepting for negative those materials which in doses of at least 3 ml. failed to produce vomiting in at least 2 previously unused animals), we undertook a study of the chemical and physical properties of the food poisoning toxin. As a preliminary step to chemical fractionation, with Dr. Favorite [17] we developed a simplified medium and method for producing highly potent enterotoxin. This medium is extremely simple and contains only dialyzable substances; acid casein hydrolysate, glucose, vitamin B_1 and nicotinic acid, none of which substances are antigenic or give a positive Biuret reaction. No agar was found necessary, thus eliminating another substance which we found to be antigenic when inoculated in rabbits. Except where otherwise stated this medium was used in all the experiments described below.

Dialysis Experiments—

Jordan and Burrows [18] and Minett [4] reported enterotoxin to be dialyzable. We have confirmed this repeatedly, using cellophane membranes and sacs, but noticeable losses occurred at some point during the procedure. However, at no time were we able to detect any enterotoxin in the dialysates, even after applying hydrostatic pressure to the bag contents and concentrating the test material by evaporation. Evidence is conclusive, we consider, regarding the large size of the toxin molecule.

Heat Stability—

It has been repeatedly demonstrated by many workers as well as ourselves that this toxin is able to withstand a temperature of 100° C. for 20 to 30 minutes with very little loss in activity.[2, 3, 5, 19]

Effect of Acid and Alkali—

Although Jordan [18] and Borthwick [20] report to the contrary (the tests which they employed cannot be accepted as reliable or specific in the light of present knowledge), Minnett [4] found enterotoxin resistant to HCl at a pH of 5.0. *In vivo,* since man is susceptible to this toxin taken by mouth, it would seem probable that it would be resistant to an even lower pH, similar to that found in the stomach. Using acetic acid to lower the pH of a culture toxin to pH 4.5 we found the toxin to be present and active in the supernate after incubation for 24 hours at 37° C. Similar treatment with NaOH at a pH of 8.0 to 8.2 appeared to have no deleterious effect.

Effect of Enzymes—

Although Minett [4] reports that a *formalinized* filtrate containing enterotoxin was inactivated by trypsin in 4 hours at 37° C. we have twice found *crude* toxins to be unaffected by this enzyme. The toxin was placed in a dialyzing membrane with 2 per cent trypsin and the reaction adjusted to pH 8.0 and this in turn suspended in a buffer solution of pH 8.0. After 48 hours' incubation at 37° C. the other toxic factors had been entirely destroyed but the material produced vomiting in

all of 4 cats inoculated by the intravenous route. Three new cats inoculated with 3 to 3.8 ml. of culture medium which had been subjected to an identical digestion with trypsin showed no reaction following inoculation.

In a similar manner to that used for the digestion with trypsin, 2 per cent pepsin in a pH of 4.3 was permitted to act for a period of 24 hours at 37° C. A control of uninoculated culture medium was similarly digested. Tests for hemolysins to rabbit cells showed these to have been completely destroyed by the digestion, but intravenous cat tests indicated that pepsin failed to digest the enterotoxin.

Effect of Fat Solvents—

Davison and Dack [11] found enterotoxin insoluble in chloroform, although Jordan and Burrows [18] reported solubility in both chloroform and ether. We were unable to find any active enterotoxin in ether extracts, but found it in the residual extracted culture filtrate. Davison and Dack [11] report that they found enterotoxin to be insoluble in alcohol, and that it was occasionally precipitated in 50 per cent and in 76 per cent alcohol. They, however, were unable consistently to duplicate their results. We have been able repeatedly to precipitate enterotoxin with ethyl alcohol, both from crude casein hydrolysate toxin and from infusion broth toxins. In experiments where the toxin had been first dialyzed, 0.8 per cent NaCl was added as an electrolyte; otherwise no adjustment was made of electrolyte or of pH. In all instances two volumes of 95 per cent ethyl alcohol were added to one volume of toxin. This was stored at 2° C. for from 24 to 48 hours and the sediment collected by centrifugation and redissolved in normal saline.

Precipitation by Ammonium Sulfate—

Davison and Dack [11] report consistent results with ammonium sulfate treatment of toxic material. In their hands, 50 per cent saturation failed to precipitate enterotoxin, while complete saturation did so effectively. Previously, in attempting to separate various hemolysins by fractional precipitation with ammonium sulfate, we had found that practically all detectable *alpha* and *beta* hemolysins were precipitated by 65 per cent saturation. When 75 per cent saturation was employed with enterotoxic filtrates all other toxins were precipitated but enterotoxin was found to remain in solution. Sterile medium and cultures of nonenterotoxigenic staphylococci treated in a similar manner invariably failed to produce vomiting in cats.

Purification of Toxin by Combined Ammonium Sulfate and Alcohol Treatment—

A combination of the two previous procedures was next employed, precipitating out the known antigenic hemolytic and dermonecrotic substance with ammonium sulfate, then precipitating the enterotoxin from the dialyzed supernate by means of alcohol. This procedure was carried out twice with toxins prepared from the " C.E." strain (originally very low in hemolytic titer) in casein hydrolysate medium and as a control repeated on a toxin from strain " W-46 " (nonenterotoxigenic). The final precipitate in each case was redissolved in a volume of distilled water equal to about 40 per cent of the volume of the original toxin. For inoculation 0.8 per cent NaCl was added. Although considerable loss occurred, a reasonable titer of toxin was found in each preparation originally containing enterotoxin (2.0 ml. to 6.5 ml. produced vomiting) and the control material produced no reaction.

The second preparation with the enterotoxigenic strain was subjected to certain quantitative and qualitative

chemical tests. The total solids were found to be 1.8 mg. per ml. A micro-Kjeldahl done in duplicate on 1 ml. of the final solution showed no detectable nitrogen. From the appearance of the acid digested residue, considerable carbonaceous material was obviously present. The Biuret test was entirely negative and the Molisch test was positive. After hydrolysis for 1 hour at 100° C. in the presence of 1.5 per cent HCl, a Folin-Wu quantitative blood sugar test was made which indicated the presence of reducing substance equal to about 0.1 mg. of glucose per 1 ml. of solution. The final solution from the first material prepared gave similar results with the Biuret and the Molisch tests. The control material from the nonenterotoxigenic strain gave an extremely small amount of precipitate on treatment with alcohol and the total solids in the final solution were found to be only 0.45 mg. per cent. The micro-Kjeldahl and the Biuret reaction employed on the active material, under the conditions of the test, would be expected to indicate the presence of protein had it represented 10 per cent of the solid material present, but very likely would not have detected smaller quantities.

Immunological Studies—

Although much has been written regarding the immunological aspects of enterotoxin,[1, 4, 6, 10, 21, 22] there is little in the way of convincing evidence for its antigenicity. Tolerance and immunity may be very easily confused, and it is possible that much that has been interpreted as immunity may have been partly or entirely due to some form of tolerance. It is possible that many so called "neutralization tests" could not be properly evaluated because of the imperfections of the test method, pit falls in interpretation, or poor suitability of the test animal.

We have as yet made no immunological test with the partially purified toxin by the ammonium sulfate-alcohol procedure, but repeated attempts with crude toxins to demonstrate specific agglutination, precipitation and *in vivo* neutralization through the use of "hyperimmune" sera from cats and rabbits failed to demonstrate any definite antigenic properties. With the possibility in mind that enterotoxin might be antigenic through a haptene linkage, and on finding that attempts to separate the enterotoxin from trypsin, after tryptic digestion, by heat and by acid precipitation were unavailing, this material containing trypsin was used to immunize a rabbit. Serum from this rabbit failed to show any precipitation or agglutination with enterotoxic material not containing trypsin, although a high titer antitrypsin serum was obtained.

DISCUSSION AND CONCLUSIONS

It would appear from the results of this study that the intravenous cat test for enterotoxin is one which can be readily applied to heat treated culture filtrates in any health department laboratory. Since adult cats or large kittens can be used, no difficulty should be encountered in acquiring and keeping the necessary animals, and each may be used three or four times before developing too great a tolerance. Negative results, however, should be confirmed by inoculation of at least two previously unused animals. A healthy cat, given a meal shortly before inoculation, should react by actually vomiting before any test is considered positive. This method of inoculation has definite advantages over the intra-abdominal route.

By use of the above test for detecting the presence of enterotoxin, certain chemical and physical studies have been made possible. The large size of the enterotoxin molecule has been confirmed. Unlike most proteins it is rela-

tively heat stable, and we have demonstrated that it is resistant to both trypsin and pepsin. Evidence from our work suggests strongly that it is nonantigenic. It is precipitated under certain conditions by alcohol and by high concentrations of ammonium sulfate, but not in a 75 per cent saturated solution of the latter. Unlike fats, it is not soluble in chloroform, ether, or alcohol. Small amounts of material originally prepared in a simple, protein-free medium, and which had undergone considerable purification by fractionation with ammonum sulfate and alcohol, were shown to be active yet, in the concentration used for inoculation, contained no detectable nitrogen by the micro-Kjeldahl method and contained a reducing carbohydrate substance. It seems probable from this evidence that the active substance is in the form of a large, complex carbohydrate molecule, and is not a protein.

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