ENTEROTOXINS OF PATHOGENIC STAPHYLOCCI: A SURVEY

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Study of the various forms of staphyloccic food poisoning is one of the urgent tasks presenting themselves in the microbiology of sanitation and food. These illnesses comprise the largest segment of all forms of bacterial food poisoning in a number of countries, including the USSR, the United States, Italy, Hungary and some others.

It has been established that the causes of food poisoning are the enterotoxins of certain pathogenic staphylocci (Dashenkov, 1901; Barber, 1914; Skorodumov, 1941; Rekstyn', 1941; Bamm, 1942; Birger, 1942; Turzhetskiy, 1947, 1949 and 1951; Popova and Sirotinina, 1948 and 1962; Ignatovich, 1949; Orlov, 1952; Nefed'yeva, 1955; Chistovich, 1957; Nagirina, 1957 and 1958; and others).

During the past decade researchers in a number of countries have sought to obtain an enterotoxin in purified form with the purpose of studying its precise nature. In the course of this research, with use of modern methods (electrophoresis, chromatographic fractionation, ultracentrifuging, spectral analysis in the infrared and ultraviolet, etc.) a number of various types of enterotoxins have been brought to light. It has been proposed to designate these by letters of the Latin alphabet, A, B, C, D, etc. (Casman et al., 1963). An enterotoxin is resistant to the effects of high tempe ratures and various chemical agents; this facilitates diagnosis and isolation of the enterotoxin, but at the same time makes the treatment of the corresponding food poisoning more difficult.

The enterotoxic properties of staphylococci are determined in biological experiments. Filtrates of cultures containing enterotoxins are administered parenterally or enterally in various test animals: cats (Davison et al., 1933; Stolnakova, 1959; Hammon, 1941; Hollander, 1966), monkeys (Bergdoll et al., 1959, 1961), frogs (Robinton, 1949, 1950; Chistovich, 1957), mice (Knizhnikov and Kasatkina, 1957), young dogs (Fujiwara

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Kikuo, 1955; Stolnakova and Nagirina, 1958), and also mature dogs (Nikodemusz et al., 1953), among others. At the present time, cats and monkeys are beingused as laboratory animals in the study of enterotoxins. A biological test with enterotoxins made on monkeys, even though specific, is not always adequate on account of the lowered sensitivity and extremely variable susceptibility of these animals (Surgalla et al., 1953). Cats have shown themselves more sensitive to parenteral administration of an enterotoxin; in addition, they are more readily available for experimentation (Thatcher and Robinson, 1962; Thatcher, 1966). Before enteral administration of enterotoxins in cats, neutralization in the culture filtrates is necessary (by boiling, use of antiserum or overboiled pancreatin) to control the α- and β-hemolysines, which bring on repeated vomiting in young cats, often with fatal results (this occurs within 5-10 minutes; Thatcher and Matheson, 1955; Thatcher and Robison, 1962). With use of an enterotoxin, vomiting sets in significantly later, and in no case does the animal die. Enterotoxins can also be tested on human volunteers. Sensitivity to an enterotoxin, either in man or animals, is a highly variable factor; any living creature may acquire resistance after repeated administrations (Casman, 1958).

In the earliest stages of developing methods for purification of an enterotoxin, researchers aimed at obtaining the substance in large quantities for the study of its biological properties. With this end in view, a special selection was made of various strains of staphylococci, culture media, and culturing conditions. Favorite and Hammon (1941), for example, were the first to propose the use of a medium containing hydrolysate of casein with glucose, nicotinic acid and thiamine in order to obtain an accumulation of an enterotoxin. Later on, culture media based on semiliquid calf bouillon, hydrolysate of casein, and overboiled casein with addition of large concentrations of glucose to prevent formation of α- and β-lysines were studied (Surgalla et al., 1951). Agar from calf bouillon, for a long time was considered one of the best culture for obtaining enterotoxins. Cultures in such media were grown both by the surface and by the depth method, with aeration and addition of 80 percent carbonic acid. The effectiveness of media for producing a large quantity of an enterotoxin was verified by feeding 50 ml of the superfluent liquid to young monkeys (Macaca mulatta). The appearance of vomiting within five hours was considered as an indication of the presence of a sufficient amount of enterotoxin.

All of the media referred to were deficient in one important respect—they contained a great deal of ballast protein substances, and this made difficult the separation of the enterotoxin in pure form. In 1958, Casman proposed a synthetic culture medium containing a number of necessary amino acids and casein hydrolysate. This medium was henceforth used by researchers in obtaining enterotoxins.

Other attempts have been made to purify an enterotoxin from staphylococcus cultures in semiliquid media in an atmosphere with 20 percent carbonic acid. (Fujiwara Kikuo, 1962). The filtrate, after passage through

a Chamberlain candle, was freed from ballast organic substances on an ion-exchange resin, then broken down into protein and nonprotein (carbohydrate) fractions which were studied to determine their chemical properties. The toxity of neither fraction, by itself, was significant; taken together, however, the two fractions affected test animals quite as much as did the filtrate previous to purification. On that basis it was concluded that an enterotoxin is a protein-carbohydrate complex.

In early studies made to obtain pure enterotoxins, relatively simple techniques were employed, such as filtration of the initial material, dialysis, concentration (often by simple evaporation), and sedimentation with alcohol (ethanol or methanol) at low temperatures and low pH. The enterotoxin thus prepared contained from 200 to 1,000 emetic doses. Vomiting in cats was produced through intrvenal administration of a dilute substrate. These enterotoxins were successfully used in the study of antigenic properties, and in the determination of general antigens in the precipitation reaction in gel with antienterotoxic sera (Casman, 1958).

Later on, Bergdoll (1956), Bergdoll et al. (1959), Hibnick and Bergdoll, demonstrated the possibility of obtaining purer preparations of an enterotoxin. In addition to acid and alcohol precipitation, dialysis, adsorption with various ion-exchange resins, paper electrophoresis, electrophoresis in starge gel, and some other methods, were used. From these a purified preparation was onbained in which the presence of a single antigen could be observed (the preparation showed a single peak in study with an ultracentrifuge). Minimal emetic dose of the preparation for producing vomiting in monkeys corresponded to 1 μg of nitrogen. In the composition of the enterotoxin, 17 amino acids per one enterotoxin molecule were discerned.

With use of purified enterotoxins from the S-6 and Ni. 196E staphylococcus strains, monospecific precipitating sera were obtained (Bergdoll et al., 1959). These sera were used for diagnostic purposes in the agar precipitating reaction, with enterotoxins from numerous strains isolated from food products, in the study of 13 cases of poisoning of staphylococcic origin.

In the agar precipitation (Oudin's method), the ratio of antigen to antibody was determined at the point of equivalence; this was shown to be 2-3 times greater than for the neutralization reaction of the enterotoxin in tests with monkeys.

The formation of enterotoxins from staphylococcus cultures was studied by Sugiyano et al. in 1960, with use of Petri dishes with agar containing antiserum to enterotoxin from staphylococcus S-6, with which the tested culture was seeded. Around the matured colonies were formed precipitation zones depending on a relationship in cell colonies producing (or not producing) the enterotoxin. The authors considered this a direct method of demonstrating various antigenic types of enterotoxins.

The same method was used to separate the S-6-R variant from the G-6 strain, which does not produce an enterotoxin. It was found also that the No. 196E strain forms an enterotoxin which is not identical to that from the S-6 strain.

Assuming the enterotoxin to be pure, the researchers obtained anti-enterotoxic sera which were used for direct observation of enterotoxin in food infected with staphylococci.

Fujiwara Kikuo in 1962 concentrated γ-globulin from immune sera and used it in tests on counter diffusion in agar (with Oudin's method) in order to observe the enterotoxin present in extracts of Japanese dishes which had been contaminated with staphylococci.

Hall et al. in 1963 demonstrated the possibility of quantitative determination of purified staphylococcus enterotoxin added to food (vegetables, meat); the gel diffusion method was used.

Casman and Bennett in 1965 described extractive and serological methods for quantitative observation of enterotoxins A and B in food products. In their work, both foods deliberately contaminated with enterotoxins and others known to have figured in cases of food poisoning, were used. The efficacy of the methods used was verifiable through determination of unidentified enterotoxins.

The first high-purity enterotoxin belonged to group B; it was obtained by Bergdoll et al. in 1959, from the S-6 strain. A number of researchers (Fujiwara Kikuo, 1962; Frea et al., 1963; Baird-Parker and Joseph, 1963; Hollander, 1966) were successful in using the methods devised by Bergdoll and his colleagues, as well as some original ones of their own. Shantz et al. in 1964 simplified the production of enterotoxin B by reducing the purification process to adsorption on ion-exchange resins. The amount of purified enterotoxin B, in powder form, amounted to 50-60 percent of the original culture. The snow-white, downy powder obtained was readily soluble in water; it contained no lipids, carbohydrates or nucleic acids; the nitrogen content was 16.1 percent. The results of spectral and sedimentation analysis showed that enterotoxin B is a simple protein, consisting of one peptide chain, and having a molecular weight of 35,000. Eighteen different amino acids were revealed by analysis. More than 45 percent of all the amino acids consisted of asparic acid, lysine and tyrosine. Serological study showed a high degree of homogeneity and purity of the preparation (99 percent or higher). The purified enterotoxin B induced vomiting, or only diarrhea, in monkeys in 50 percent of cases; this was achieved with doses as small as 0.1-0.3 μg/kg in the case of intravenous administration, and as small as 1 μg/kg in the case of oral administration.

Bergdooo et al. in 1965 obtained a new purified enterotoxin which they associated with Group C. In subsequent studies, Borja Concordia and Bergdoll (1967, 1969), and also Avena Remedios and Bergdoll in 1967, studied some of

the biochemical properties of toxin C obtained from strains No. 137 and 361. The purified enterotoxin was found to be a protein with isoelectric point 8.6. Administration of 5 mg of preparation in the stomach produced specific poisoning in 12 out of 24 monkeys; a 0.5 mg intravenous administration produced symptoms of poisoning in 7 out of 9 animals. The preparation contained 16.2 percent of nitrogen, and no carbohydrates, nucleic acids or lipids.

A method of preparing and purifying enterotoxin A which guaranteed a 35 percent yield of pure product was worked out in 1966 (Fun Sun Chu et al.). This purified enterotoxin A is a simple protein with molecular weight of 34,700 and isoelectric point 6.8. Nitrogen content is 16.5 percent. The toxicity was studied in tests with young monkeys (body weight 2-3 kg), to which were administered 50 ml of the purified enterotoxin in the stomach, or 2 ml intravenously. Vomiting within 5 hours was considered a positive reaction.

Kato Erichii et al, in 1967, separated enterotoxins A, B, C_1 and C_2 from culture filtrates of various staphylococcus strains; these were not identical in antigenic properties. The purified preparations (on ion-exchange resins and carboxymethylcellulose) were molecular weights ranging from 30,000 to 34,700; sedimentation and diffusion constants varied, as viscosity; precipitation occurred at various pH values. Enterotoxin A readily lost its antigenic properties upon heating, ceasing to produce precipitation in agar with an homologous serum. Enterotoxin B was more stable, and C_1 and C_2 were intermediate as regards heat-resistance.

The same year Casman et al. established the possibility that staphylococci may produce a previously unknown enterotoxin, which the designated as enterotoxin D.

This particular enterotoxin was obtained from a strain which produced no A, B or C, and which was absent from the growth products of nonenterotoxic strains. Partially purified enterotoxin D produced vomiting in cats, and its biological activity was neutralized only by a serum containing specific antibodies, and not by sera A, B or C.

Beginning with 1966, studies appeared which had profoundly utilitarian significance both for the clinical study and the diagnosis of illnesses; these were also important in epidemiology.

Kienitz, in 1966, in 24 cases of enterocolitis associated with food poisoning, separated staphylococcus strains which produced an enterotoxin. Using the precipitation reaction, together with the corresponding antisera, he was able to determine the frequency with which enterotoxins A and B were present in cases of food poisoning and enterocolitis. Of the 15 patients with enterocolitis, 9 had enterotoxins A and B, 5 had enterotoxin B, and only 1 had enterotoxin A. Of the 9 patients with food poisoning, 6 had

enterotoxin A, and three both A and B. Hallander and Körlof, in 1967, studied 50 staphylococcus strains, isolated in autotoxins from wounds. Of these, 18 were able to produce enterotoxins A and B. It was established that 6 out of 7 cases of enterocolitis were caused by staphylococci which produced enterotoxin A.

In 1967, Casman et al. studied 80 staphylococcus strains obtained from food poisoning cases, in order to determine enterotoxins of various immunological types. Enterotoxin A, by itself, was found in 49 percent of cases, and, in combination with D, in 25 percent; enterotoxin D, alone, was present in 7.6 percent of cases; enterotoxin B, in 3.8 percent; enterotoxin C, in 2.5 percent; A and B combined, in 1.25 percent; and combinations of B and C, C and D, and A, B and D, in about 2.5 percent. It is of interest that strains producing enterotoxin D were obtained from only milk and frozen foods, while those producing enterotoxins A and D, were obtained from cases of food poisoning, or from the nasopharynges of well persons.

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Tests/made for serological identification of staphylococcic enterotoxins of strains obtained from quality foods and from products producing food poisoning. From poisoning-producing cheese, Donelly et al. (1967) were able to separate staphylococci in 77 cultures, enterotoxin A being produced by 7 of these; while from 155 strains drawn from pure cheese, 9 produced the enterotoxin. In the case of two strains, an enterotoxin was obtained which did not cause precipitation in the presence of antisera A and B. These staphylococci, evidently, produced enterotoxins of other types.

In 1966, Hallender and Laurell, from an S-6 staphylococcus culture, produced an immunologically homogeneous preparation of enterotoxin, with immunization by which autotoxic sera were obtained. Using these sera, the authors studied 50 strains of pathogenic staphylococci, these being obtained from corpses; also studied were 96 strains from wounds, and 148 strains from feces. Enterotoxin B was observed in 18 of the cultures obtained from corpses; and in 21 of those obtained from other sources. The majority of the cultures producing enterotoxin B belonged to phagotype 47; these produced various pathological processes, many of which proceeded along with the poisoning syndrome. It is possible that enterotoxin B produced a graver course of the illness. Studies of flare-ups in breast-surgery hospitals associated with staphylococci of phagotype 75/77 (these produce enterotoxin B, 18 of 24 patients vulnerary infection and bronchopneumonia; in addition, 4 developed enterocolitis in addition to their basic illness. Nine of the 24 patients died.

In 1966-1967 attempts were made to cut down the period of time needed for observation of an enterotoxin. To detect enterotoxin B in food products, Genigeorgis and Sadler i. 1966 made successful use of a specific antienterotoxic, to which was added fluorescinisothiocyanate. The method of fluorescing antibodies made it possible to determine a small quantity of the enterotoxin (less than 1 or 0.05 mg/ml), and the whole process required

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only 4-5 hours.

Morse et al. in 1967 proposed the microtitration determination of the staphylococcus enterotoxin B, by means of retarding hemagglutination, which would also permit observation of a small quantity of the enterotoxin. The method consisted in retarding with an antienterotoxic serum the agglutination of a suspension of sheep erythrocytes first treated with Formalin and tannin and and loaded with a purified toxin. This means of determination required only three hours.

For quick separation of enterotoxin B in the culture liquid of a staphylococcus, the method of hemagglutinating Formaline-treated sheep erythrocytes prepared with monodiazotized benzidine has ben used (Johnson et al., 1967). Parallel to this was made a determination of the enterotoxins of the hemagglutination delay reaction and diffusion in agar. The sensitivity of the delay reaction and the diffusion reaction was found to be the same, but the reaction time was calculated as only several hours, whereas the agar diffusion reaction certainly requires 24 hours. The hemagglutination delay reaction was used to reveal 8 cultures with enterotoxins out of a total of 68 cultures. In any case, the advantage of the delay reaction ceased to be of interest following development of the method of processing an enterotoxin with trypsin, or by boiling for an hour. Enterotoxin and the amount of 0.033 γg per 0.25 ml of culture liquid producedthe hemagglutination delay reaction

Riemann in 1967, using immunofluorescent microscopy, was able to observe enterotoxin B in food products. This method is quite sensitive; the enterotoxin was detected in concentrations lower than 0.05 γg/ml.

Thus, as is evident from the foregoing, during the past 10-15 years a large body of research devoted to the nature of the staphylococcus enterotoxin has been undertaken. Four enterotoxin types (A, B, C and D) have been isolated in purified form; antisera have been developed for use in diagnosis. Serological diagnosis is used in conjunction with phagotyping. Attempts are being made to find accelerated methods of observing enterotoxin (hemagglutination delay reaction, immunofluorescent microscopy, and others).

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