Enterotoxin Production by *Staphylococcus aureus* Strains Isolated from Cases of Chronic Osteomyelitis

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Of 264 *Staphylococcus aureus* strains selected at random from 3,978 strains isolated from chronic osteomyelitis patients, 79 were found to produce enterotoxin. A majority of the strains (65%) were nontypable by phages belonging to the international phage set for human strain typing. The significance of these strains and their circulation among the population is discussed, especially from the standpoint of their possible role in the development of osteomyelitis.

Staphylococcus aureus strains produce many proteins, some of which are responsible for a syndrome in humans generally known as staphylococcal food poisoning. The latter proteins are called enterotoxins (A, B, C, D, E) and are differentiated on the basis of their antigenicities (5). The illness (nausea, cramps, vomiting, and diarrhea) is usually caused by consumption of foods in which staphylococci have grown and produced enterotoxin. It differs from enterotoxic substances produced by certain gram-negative bacteria in that it does not give a positive reaction in the ileal loop test. Much work has been done on its mode of action (1), but the exact site has not been located. It is not known what proportion of diarrheal disease is caused by enterotoxigenic strains (9), some of which may colonize the digestive tract and/or the nasopharynx. An answer to this question depends on the availability of data on the prevalence of enterotoxigenic staphylococci as well as on specific immunity and/or the protective mechanisms acting against staphylococcal infection in the general population. It is possible to obtain such data because reagents and methods are available for the detection of enterotoxigenic strains by any microbiological laboratory.

In this paper the results of a study on *S. aureus* strains isolated from patients suffering from chronic osteomyelitis are presented. This study was undertaken to determine whether enterotoxigenic staphylococci are associated with illnesses other than enterotoxigenic.

MATERIALS AND METHODS

***S. aureus* strains.** *S. aureus* strains were isolated from 1,201 patients with chronic osteomyelitis, predominately of staphylococcal etiology. The patients, from different regions of Czechoslovakia, were treated

as outpatients at the Research Surgical Unit of the Institute of Clinical and Experimental Medicine, Prague, Czechoslovakia. From a total of 3,978 strains isolated from lesions, throats, and nasal passages, 264 were selected for this study, 204 from lesions and 60 from throats or nasal passages of the same patients from which the 204 were isolated. The latter strains were identical to those isolated from the lesions.

Staphylococcal enterotoxin production. The cellophane-over-agar method (6, 7, 10) was used for growing of the staphylococci to obtain materials for testing for enterotoxin. The medium contained brain heart infusion, 3.7%; enzymatic hydrolysate of casein, 3.0%; nicotinic acid, 1.2 mg/liter; calcium pantothenate, 0.5 mg/liter; thiamin, 0.4 mg/liter; and arginine, 125 mg/liter. Cultures were incubated at 37°C for 48 h and then washed from the cellophane with 1 ml of distilled water containing merthiolate (1:10,000) and kept at 4°C overnight. The cultures were centrifuged, and the supernatant fluids were freeze-dried. The dried materials were redissolved at a 10-fold concentration in 0.02 M sodium phosphate, pH 7.4, in 0.9% NaCl containing 1:10,000 merthiolate (PBS).

Enterotoxin standards. The enterotoxin standards were crude enterotoxins standardized against specific antisera in the Food Research Institute or partially purified enterotoxins prepared in the Institute of Hygiene and Epidemiology. The dried materials were dissolved and diluted in PBS for use in the microslide immunodiffusion tests.

Enterotoxin antisera. Specific antisera to the enterotoxins were prepared in rabbits at the Food Research Institute with highly purified enterotoxins (2) and at the Institute of Hygiene and Epidemiology with partially purified enterotoxins (11). The latter antisera were treated with culture supernatant fluids from non-enterotoxigenic staphylococci to remove antibodies not specific for the different enterotoxins. Both sets of antisera were used in the microslides at those dilutions producing the sharpest precipitation lines which approximated the points of equivalence of the systems under titration (1 to 20 µg of enterotoxin per ml of PBS).

Enterotoxin detection. A modification of the microslide immunodiffusion method was used for detection of the enterotoxins in the culture supernatant fluids (4). Agar was placed on a microscope slide, and holes were cut in the agar in place of using a template. The well size used was 3 mm in diameter with a distance of 2.5 mm between the center and peripheral wells, which allowed for five peripheral wells (Fig. 1). The antisera were placed in the center well with the enterotoxin standards, and unknowns were placed in the outer wells. Positive reactions were indicated by coalescence of lines formed by the unknowns with the lines formed by the enterotoxin standards.

Phage typing. The international set of phages for typing *S. aureus* strains of human origin plus phages 92 and 187 were employed with standard test conditions (3). Cultures were typed at both routine test dilution and 100 routine test dilutions.

RESULTS

Enterotoxin production by *S. aureus* strains. Of the 264 *S. aureus* strains tested, 79 produced enterotoxin. Forty-five (57%) produced enterotoxin A, 14 (17.7%) produced enterotoxin B, 14 (17.7%) produced enterotoxin C, 2 (2.5%) produced enterotoxin D, 3 (3.8%) produced enterotoxins A and B, and 1 (1.3%) produced enterotoxins A and D. Concentration of the supernatant fluid from 47 strains was not necessary to detect the enterotoxin. Those which gave negative results were tested in the concentrated form three to five times. In cases where the results were questionable, the following arrangement was used for retesting: well 1, standard enterotoxin; wells 2 and 4, concentrated culture supernatant fluid under question; wells 3 and 5, PBS. If the reactions obtained from the material in wells 2 and 4 are identical to the control, the results are considered positive. Most of the enterotoxigenic strains were isolated from lesions rather than from the nasal passages or throats of the patients.

Both sets of antisera were used in the tests to determine how the set prepared in Czechoslovakia compared with the set prepared in the Food Research Institute, a primary source of enterotoxin reagents. The results with the two

sets were identical both when tested together and separately, except that the antibody titer was lower in the antisera prepared in Czechoslovakia (Fig. 1). This did not affect the results obtained, indicating that these antisera could be used to replace the ones provided by the Food Research Institute.

Phage typing. Results of the phage typing on the enterotoxigenic strains are given in Table 1. One strain was not included because it was coagulase negative. A majority of the strains were nontypable (65%), whereas eight were lysed by both group I (79) and group III (53) phages. Five strains were lysed by phages 92, 96, and 187. Other phages found to lyse at least one strain were: 29, 52, and 79 (group I); 3C, 55, and 71 (group II); and 53, 75, 77, 83A, 84, and 85 (group III). The phages reacting most frequently were 53 (10 strains), 79 (8 strains), 84 (5 strains), and 85 (4 strains). There was no relationship between the phage and enterotoxin types.

DISCUSSION

The etiology of most staphylococcal infections is not known. It is difficult to associate infections such as osteomyelitis with any given substance of the many produced by the staphylococci, including the enterotoxins. The strains isolated

FIG. 1. Enterotoxin A production of *S. aureus* strains. Wells 1 and 3: standard enterotoxin A from the Food Research Institute; well 2: strain 1; well 4: strain 2; well 5: strain 3; center well 1A: anti-enterotoxin A (Food Research Institute, Madison, Wis.) center well 1B: anti-enterotoxin A (Institute of Hygiene and Epidemiology, Prague, Czechoslovakia).

TABLE 1. Phage typing of coagulase-positive *S. aureus* strains

Enterotoxin type	No. of strains lysed by phage group:						
	I	II	III	I + III	Unclassified	Not typable	Total
A			4	8	1	31	44
B		3	4		2	5	14
C	1				1	12	14
D					1	1	2
AB			1			2	3
AD			1				1
Total	1 (1.3) ^a	3 (3.8)	10 (12.8)	8 (10.3)	5 (6.4)	51 (65.4)	78 (100)

^a Data in parentheses are percents.

from the osteomyelitis patients apparently could not be classed as so-called "hospital strains" because they differed widely in their enterotoxin and hemolysin production, phage type, antibiotic resistance patterns, etc. This would be expected because the patients came from widely separated areas in Czechoslovakia and had not had prior contact with the hospital. The fact that only 30% of the cultures isolated from the patients suffering from osteomyelitis were enterotoxigenic indicated that enterotoxin production is an incidental characteristic of these strains.

The primary cause of enterotoxigenosis in humans is the ingestion of food containing enterotoxin, but in some instances this ailment occurs when the organisms grow uninhibited in the intestinal tract after antibiotic therapy (12). There was no evidence of enterotoxigenosis in the osteomyelitis patients in this study, nor to our knowledge is enterotoxigenosis associated with other staphylococcus-related infections even though enterotoxin may be produced in the infections. This is indicated when higher titers of antibodies specific for the enterotoxin associated with the staphylococci responsible for various infections are present in the sera of those suffering from the infections (8). Enterotoxigenosis results if sufficient quantities of enterotoxin are injected into the circulatory system (1); hence, apparently insufficient quantities of enterotoxin get into the circulatory system from infections to cause enterotoxigenosis.

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