Use of the Aggregate-Hemagglutination Technique for Determining Exo-Enterotoxin of Bacillus cereus

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The possibility of using the aggregate-hemagglutination technique for detection of Bacillus cereus exo-enterotoxin in foodstuffs and culture media is shown. A 0.004-μg quantity of enterotoxin per ml can be detected by this method.

The enterotoxigenicity of Bacillus cereus was at one time attributed to lecinthase C production by the bacterium (3, 6, 16), although some workers (13, 15; A. Ottolenghi, S. Gollub, L. Lisbinsky, and A. Ulin, Fed. Proc. 22:1002, 1963) suggested that B. cereus might produce a toxin that was not identical to lecinthase. A highly purified enterotoxin was recently obtained from B. cereus (7). This toxin does not possess lecinthase or hemolytic activity; it is lethal for mice, rabbits, rats, and cats (10, 11); and it displays enterotropic properties when injected into cats (8, 11).

Various methods have been developed for the quantitative determination of bacterial enterotoxins; these methods differ both in sensitivity and in principle. One of the most sensitive methods of antigen determination is the passive hemagglutination-inhibition reaction (2, 4). In recent years a new and more sensitive technique, aggregate-hemagglutination, has been proposed for detection of antigen content. It is based on the use of erythrocytes sensitized by aggregated proteins of immune serum (17–19). Antibodies are introduced into three-dimen-sional protein complexes, so that the active sites of the antibodies are spaced some distance from the surface of the erythrocytes. Thus, the active sites become readily accessible to antigenic determinants.

The reaction of aggregate-hemagglutination was used previously for detecting antigens of different origin (1, 20). The simplicity and sensitivity of the aggregate-hemagglutination technique make it convenient for detecting of small quantities of bacterial antigens for diagnostic purposes. Data on the enterotoxin of B. cereus, detected by the aggregate-hemagglutination technique, are given in this communication.

MATERIALS AND METHODS

Cultures and toxin production. Toxin-producing B. cereus 96 was grown in a medium containing fermented casein hydrolysate and 100 to 150 mg of amino nitrogen per ml (9). The growth medium contained (grams per liter): glucose, 5.0; NaHCO₃, 5.6; CaCl₂2H₂O, 0.0147; MgSO₄·7H₂O, 0.0197; K₂HPO₄, 0.174; KH₂PO₄·0.136; MnSO₄·H₂O, 0.00172; adenine sulfate, 0.002; uracil, 0.007; thiamine hydro-chloride, 0.0001; glycine, 0.0003; tryptophan, 0.00024; and cystine, 0.00024. The pH was adjusted to 6.8 to 7.0. The standard conditions for incubation were as follows: 37 C for 16 to 24 h on a shaker at 110 rpm. Other Bacillus strains used in this study were grown under analogous conditions. Strains of B. mesen-tericus (B. cereus), B. cereus var. mycoides, B. anthracis, B. thuringiensis, and B. subtilis were obtained from the Tarasevitch State Control Institute, Moscow. B. megaterium strains 207, 216, and 337 were kindly supplied by E. N. Levina. All the above microorganisms were grown for 24 h at 37 C, as was described previously (9). The producer of enterotoxin A, Staphylococcus aureus 100, was obtained from the collection of A. C. Baird-Parker. S. aureus 243, the producer of enterotoxin B, was kindly supplied by E. P. Casman. S. aureus strains 100 and 243 were grown in Casman medium (5) for 24 h at 37 C.

Escherichia coli was kindly supplied by V. G. Petrovskaya; it was grown in peptone broth for 24 h at 37 C. The culture filtrates of Shigella dysenteriae and Clostridium perfringens were obtained from V. P. Solodovnikov; those of Vibrio cholerae were from G. D. Kobrinsky. The enterotoxin of B. cereus, produced and purified by the method of Ezechuk and Fluer (7), was isolated by ammonium sulfate treatment followed by fractionation in Bio Gel P-150 in 0.015 M NaCl (pH 7.0) (7, 8, 11).

Foodstuffs were inoculated with 10⁴ cells of B. cereus 96 per g and then were kept at 4, 24, or 28 C for 16 h. Solid, infected foodstuffs were ground in a microgrinder and suspended in saline (1 g of food stuff per 1 ml of saline). After filtration and centrifugation of the suspension to remove undissolved fragments, filtrates were analyzed for enterotoxin.
The protein concentration of the purified enterotoxin was determined by the method of Lowry et al. (14). The lethal activity was determined by injections of the toxin preparation or the culture filtrates into the tail veins of white random-bred mice (weight, 15 to 20 g); responses of the mice were estimated after 30 min. The minimum dose of enterotoxin causing a reaction in response to intravenous injection in cats (weight under 4 kg) was estimated after 1 h. Various bifunctional agents may be used for aggregation of immune serum proteins, in particular the stabilized tetraazotate of 4,4'-diaminodiphenylamine and glutaraldehyde. Glutaraldehyde was used at all stages of present work by the method of Gorina and Olovnikov (12).

**Antiserum.** To obtain antisera to a purified preparation of *B. cereus* enterotoxin, three subcutaneous injections of toxin in the footpads and in four sites on each side (5 mg per rabbit, mixed with Freund incomplete adjuvant) were made at 1-month intervals. Two weeks after the third injection, antiserum with an antibody content of about 1.5 mg/ml, as determined by a slight modification of the neutralization test (21), was obtained for use in the experiment. The antiserum had a 1:64 titer by the Ouchterlony technique, where serum double dilutions were prepared against enterotoxin (protein concentration in enterotoxin preparation, as determined by the method of Lowry [14] was 1.5 mg/ml).

**Aggregate-hemagglutination technique.** Stabilization and activation of erythrocytes were done by the method of Gorina and Olovnikov (12). Two volumes of 0.9% saline were added to 1 volume of packed erythrocytes, and then a 25% aqueous solution of glutaraldehyde (Merck) was added to obtain a final concentration of 0.25%. After incubation for 3 h at 37°C, the erythrocytes were washed four times in saline and then resuspended in saline. Glutaraldehyde was again added to a final concentration of 0.25%, and the erythrocytes were refrigerated as an 8% suspension until use. For aggregation, 0.08 ml of 2.5% glutaraldehyde was added to 1 ml of antiserum, and after 1 h of incubation at 37°C the aggregated antiserum was used for sensitization. A 1-ml volume of the 8% suspension was removed from the glutaraldehyde-saline suspension, washed with saline, and suspended in aggregated immune serum (concentration of 8%). After 90 min of incubation at 36°C and then during the 30-min period at room temperature, sensitized erythrocytes were washed three times with saline, and a 1 to 2% suspension of red blood cells in saline, preserved with merthiolate (1:10,000), was prepared (12). Agglutinations were performed by the Takatsy microtitration procedure, with 0.2% normal rabbit serum as diluent. The results were estimated after incubation for 4 h at 37°C. Erythrocytes, sensitized by aggregated proteins of normal rabbit serum, were used as controls.

**RESULTS AND DISCUSSION**

In this work we attempted to detect microquantities of *B. cereus* enterotoxin in cultures and foodstuffs by the aggregate-hemagglutination technique. The sensitivity of this method exceeded that of the Ouchterlony procedure by about 1,000 times. It is suggested, therefore, that it is a convenient means for diagnostic determination of bacterial enterotoxin.

The antiserum used was not monospecific and gave three lines of precipitation in gels against the enterotoxin preparation used for immunization. One line corresponded to a thermolabile antigen, and two other lines corresponded to thermostable antigens. The thermostable antigens seemed to contain polysaccharide; only traces of antibodies to these antigens were present in the antiserum. Before heating, the enterotoxin preparation (protein concentration of 1.5 mg/ml; see Table 1) with the thermolabile compound gave a titer of 1:262,000 by aggregate-hemagglutination and, after heat-

**Table 1. Use of the aggregate-hemagglutination technique for detection of enterotoxin of *B. cereus***

| Antigen                                      | Agglutination titers |
|----------------------------------------------|----------------------|
| Enterotoxin of *B. cereus*, 1.5 mg/ml        | 1:262,000           |
| Enterotoxin of *B. cereus*, heated 30 min    | 1:2                  |
| Culture filtrate of *B. cereus*              | 1:32,768            |
| Culture filtrate, heated 30 min              | 1:8                  |
| Culture filtrate, 100 C                       | 1:8                  |
| Culture filtrate of *B. anthracis*           | 1:4                  |
| Culture filtrate of *B. anthracis*, 30 min   | 1:4                  |
| Culture filtrate of *B. megaterium* 207      | 1:16                 |
| Culture filtrate of *B. megaterium*, 207, 100 C | 1:4                 |
| Culture filtrate of *B. megaterium* 207      | 1:2                  |
| Culture filtrate of *B. megaterium* 337      | 1:4                  |
| Culture filtrate of *B. mesentericus* 1227   | 1:2                  |
| Culture filtrate of *B. subtilis*            | 1:0                  |
| Culture filtrate of *B. cereus* var. mycoides| 1:0                  |
| Culture filtrate of *B. thuringiensis* 13-1  | 1:0                  |
| *var. galleria*                              | 1:0                  |
| Culture filtrate of *S. dysenteriae*         | 1:4                  |
| Culture filtrate of *E. coli*                | 1:2                  |
| Culture filtrate of *C. perfringens*         | 1:2                  |
| Culture filtrate of *V. cholerae*            | 1:2                  |
| *Staphylococcus* enterotoxins purified in Bio Gel P-60: Type A | 1:2 |
| *Type B*                                     | 1:2                  |
ing at 100 C for 30 min, it gave a titer of 1:4. The antibody titer against thermolabile antigen by the Ouchterlony procedure was 1:64, and antibody titers against two thermostable antigens were 1:4. Thus, at dilutions of the antiserum 1:8 or higher, one can obtain a single precipitation line corresponding to the thermostable antigen.

Closely related aerob microorganisms of the Bacillus type, such as B. anthracis, B. megaterium, etc., are known to have common thermostable antigens polysaccharide in nature. The antiserum that we used in aggregate-hemagglutination gave cross-reactions with two closely related microorganisms, B. anthracis and B. megaterium, yielding titers of 1:8 to 1:16. Other experiments (data not shown) proved by gel precipitation that both thermostable components of B. cereus enterotoxin preparations are immunologically identical to the thermostable antigens of closely related Bacillus species. The aggregate-hemagglutination technique for detecting B. cereus toxin was sensitive down to 0.004 mg/ml.

The erythrocyte diagnosticum used is a specific one and does not give in the aggregate-hemagglutination of cross-reactions with enterotoxins produced by other microorganisms such as S. aureus, C. perfringens, E. coli, Shigella dysenteriae, Vibrio cholerae (Table 1).

To exclude the possibility of cross-reactions with the thermostable antigens of B. cereus, foodstuffs were heated in control experiments for 30 min at 100 C. Purified toxin and culture filtrate of toxigenic B. cereus yielded high titers (Table 1). The titers of heated and unheated preparations were uniformly low for a number of nontoxigenic Bacillus species and cultural filtrates of various other bacteria. It follows that, even if non-monospecific serum is used and the protein toxin is inactivated by heating, the specificity of B. cereus enterotoxin may be proved by this procedure.

When the purified enterotoxin of B. cereus was injected intravenously into mice, the minimum lethal dose was 300 μg per mouse (Table 2). The enterotoxic effect in cats caused by B. cereus enterotoxin was observed after intravenous injection of 70 to 80 μg/kg (live weight). For evidence of neutralizing activity of the antiserum, the enterotoxin of B. cereus was mixed with antiserum, incubated for 1 h at 24 C, and injected into mice. The mixture, consisting of 0.25 ml of toxin (about 3 minimum lethal doses) and 0.25 ml of homologous antisemum (antibody protein content, about 1.5 mg/ml), was not lethal for mice. It was possible to detect by aggregate-hemagglutination a quantity of toxin approximately 75,000 times lower than that detected by the biotests (Table 2).

| Foodstuffs         | Temp of incubation (°C) | Agglutination titers |
|--------------------|-------------------------|----------------------|
| Boiled meat        | 4                       | 1:128                |
| Raw meat           | 24                      | 1:2                  |
| Sausage            | 4                       | 1:128                |
| Raw meat           | 24                      | 1:32                 |
| Sausage            | 4                       | 1:8                  |
| Milk               | 24                      | 1:32                 |
| Tea                | 24                      | 1:2                  |

Control for specificity with uninoculated foodstuffs:

| Foodstuffs         | Temp of incubation (°C) | Agglutination titers |
|--------------------|-------------------------|----------------------|
| Boiled meat        | 4                       | 1:2                  |
| Meat broth         | 24                      | 1:2                  |
| Raw meat           | 4                       | 1:4                  |
| Sausage            | 24                      | 1:8                  |
| Milk               | 24                      | 1:8                  |
| Tea                | 24                      | 0                    |

*Cattle meat and boiled sausage were used.
The results showed the possibility of using this method for detecting enterotoxin in different foodstuffs infected by B. cereus. Beef, boiled sausages, and other foodstuffs were incubated at 4, 24, or 28°C for this purpose. Uninoculated foodstuffs incubated at the same temperature as inoculated foodstuffs served as controls.

The enterotoxin of B. cereus was detected by the aggregate-hemagglutination test in boiled meat (titer, 1:4,096), meat broth (1:512), and boiled sausage and raw meat (1:128) when these foodstuffs were kept for 16 h at 24°C (Table 3). Increase in incubation temperature of foodstuffs inoculated with B. cereus up to 28°C resulted in increases of the agglutination titers by 8 to 30 times. Only small quantities of enterotoxin (titers at 1:8) were found in the same foodstuffs kept at 4°C. Such reactions were considered to be nonspecific.

Controls were as follows. Erythrocytes coated with aggregated proteins of immune serum were agglutinated with extracts of inoculated foodstuffs or with cultural media at titers of 1:2 to 1:16 (Table 3). Erythrocytes coated with aggregated proteins of serum were not agglutinated by the same materials. Controls for nonspecific hemagglutination caused by noninfected products and by cultural filtrates of closely related strains, as well as controls with erythrocytes coated with aggregated proteins of nonimmune serum, were 1:2 to 1:16.

The aggregate-hemagglutination technique gives reproducible results and does not require much time for the reaction. Sensitized erythrocytes persist for a month without loss of activity, and thus this reaction is convenient for practical detection of B. cereus enterotoxin in foodstuffs and culture media.

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