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Alteration of Vascular Permeability in Rabbits by Culture Filtrates of *Bacillus cereus* and Related Species

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Crude culture filtrates of strains of *Bacillus cereus*, *B. thuringiensis*, and *B. mycoides* caused an increase in vascular permeability when injected intradermally into rabbits. The time course of the change in permeability was determined, and could easily be distinguished from a more transient effect induced by purified (from *B. cereus*) phospholipase C. The properties of the responsible factor were found to be similar to those reported for the guinea pig dermal factor and the ileal loop fluid inducing factor, namely: synthesis by vigorously aerated, logarithmically growing cells; inactivation by heating at 56 C; non-dialyzability; precipitation with ammonium sulfate; and antigenic characteristics. Permeability factor was not related to either the phospholipase C or the hemolysin produced by *B. cereus*. Activity of this *B. cereus* toxin, as measured in the vascular permeability and ileal loop assays, can easily be quantitated, but the greater simplicity, reliability, and economy of the vascular permeability test make it the method of choice for screening cultures or following toxin purification.

The role of *Bacillus cereus* as an agent of food poisoning has received increasing attention and has been the subject of a recent review (6). Spira and Goepfert (9) have shown that there is a factor present in cell-free culture filtrates of *B. cereus* which causes fluid to accumulate in ligated intestinal loops in rabbits. This assay for enteropathogenicity is tedious, time-consuming, and rather cumbersome for routine application. Many of the enteric pathogens, which induce fluid accumulation in the ligated intestinal loop, also produce a factor which elicits a characteristic response when injected into the skin. Dermal assays of enteropathogenicity have been described for *Vibrio cholerae* (2, 3) and other vibrios (1), enteropathogenic *Escherichia coli* (4), and *Clostridium perfringens* (7, 10). Previously, we described the response of guinea pig skin to intradermal inoculation of crude culture filtrates of ileal loop-positive strains of *B. cereus* (5). In this communication, we report on the vascular permeability influencing activity of *B. cereus* culture filtrates, as measured by an assay system adapted from the one developed by Craig (2) to study *Vibrio cholerae* enteropathogenicity.

MATERIALS AND METHODS

Cultures. Eleven strains of *B. cereus* were used in this study. In addition, five strains of *B. thuringiensis*, four strains of *B. mycoides*, two strains of *B. lichenformis*, and single strains of *B. megaterium* and *B. subtilis* were tested. Unless otherwise noted, all experiments were performed with a single ileal loop-positive strain of *B. cereus*.

Growth media and conditions. Growth media included nutrient broth, Trypticase soy broth, and brain heart infusion broth supplemented with 0.1% glucose (BHIG). Stock and working cultures were maintained as described previously (5).

Preparation of culture filtrates. A sample (0.3 ml) from an overnight culture was added to 30 ml of culture medium (BHIG was used routinely) in a 125-ml Erlenmeyer flask and incubated for 8 h at 32 C on a gyratory shaker at 84 cycles/min. Cultures were centrifuged at 12,840 × *g* for 10 min in a Sorvall RC2-B centrifuge, and the supernatant was sterilized by passage through a Gelman 0.2-μm membrane filter.

Assay for permeability influencing activity. New Zealand white rabbits weighing 2 to 3 kg were used for the tests. The back was shaved, depilated, and marked off into 25 to 36 squares. Intradermal injections of samples (0.05 ml) were made into separate squares. Three hours after administration of the test samples, Evans blue dye was injected into the ear vein at a dosage rate of 1 ml of a 10% dye solution in physiological saline per kg of body weight. Animals were observed for up to 60 min after administration of the dye.

Assays for other activities. Tests for phospholipase C (5), hemolysin (5), and ileal loop fluid inducing factor (9) were performed as described previously.

Definition of toxic unit. A unit of toxic activity

was defined empirically in terms of the assay used. In the rabbit ileal loop (RIL) assay, a diarrheagenic unit (DU) is that amount of toxin eliciting a fluid volume-to-length ratio of 0.20. In the assay for permeability factor (PF), a PF unit is that amount of toxin eliciting an elliptical area of bluing of 7 mm^2.

Immunological tests. Antiserum was prepared by injecting the appropriate antigenic preparation on a regular schedule subcutaneously into New Zealand white rabbits. Serum was obtained after 5 weeks of inoculations. Neutralization tests were performed by mixing test substance and antiserum at a 2:1 (vol/vol) ratio and incubating the mixture at 37 C for 45 min before use in the various assay systems.

RESULTS AND DISCUSSION

Characteristics of the reaction. The culture filtrate of *B. cereus* strain B-4ac, known to be positive in both the ileal loop and guinea pig dermal assays, was used in determining the characteristics of the vascular response.

Immediately after injection of dye, the entire skin of the rabbit turned a faint gray-blue. Within 10 min, positive reactions began to appear as areas of darker blue around the sites of inoculation of the culture filtrate preparations. Bluing was usually maximal in both intensity and area within another 20 min, and was stable for at least another hour. More potent preparations also caused a necrotic reaction at the site of injection, analogous to that reported to occur in guinea pigs (5), and thus these responses appeared as a halo of dark blue surrounding a central bloody or dark-red area. The elliptical area of bluing was taken as an indication of the amount of plasma protein that had been released into the dermal tissue due to altered vascular permeability, and ranged from 4 to over 100 mm^2.

Time course of the bluing reaction. Tests performed with purified *B. cereus* phospholipase C (General Biochemicals, Inc.) at a concentration of 10 mg/ml in physiological saline indicated that this substance was also capable of causing a bluing response. The time course of the appearance of the reaction to this preparation was therefore compared with that elicited by culture filtrate (Fig. 1). If phospholipase C was injected into the animal 2 h or less before administering of dye, a prominent blue response was noted. However, an interval of 3 or more hours between injections of phospholipase and dye resulted in the absence of bluing. In contrast, a blue region was observed at the site of inoculation of crude culture filtrate injected as much as 6 h before the dye. A transient response identical to that obtained with purified phospholipase C was observed when culture filtrate of strain B-4ac was heat treated to inactivate

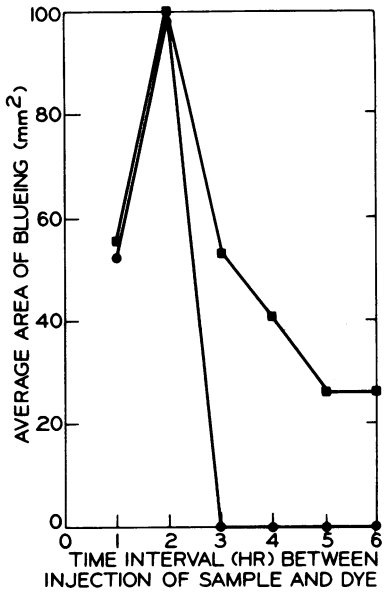

FIG. 1. *Time course of the bluing response to injection of culture filtrate* (■) *and of purified B. cereus phospholipase C* (●).

PF but not phospholipase C (discussed in a later section) and assayed. Phospholipase C in purified and unpurified form acts similarly. Thus, the permeability change induced by phospholipase C is of a much more transient nature than is the response elicted by PF present in culture filtrates. For this reason, a standard interval of 3 h was chosen between injection of test preparations and dye to preclude interference by phospholipase-induced reactions.

A similar situation of differential effects on vascular permeability was reported by Stark and Duncan (10) with *C. perfringens*. Capillary permeability was increased maximally (as determined by the size and intensity of the blue region) if pure phospholipase C was injected between 1.5 and 2.5 h before the dye. However, a blue response could be elicited by a purified enterotoxin preparation only if it was administered less than 30 min before the dye.

Quantitation of toxic activity. Cell-free culture filtrate concentrated six-fold in Carbowax was used to investigate both the PF and RIL assays as means of quantitating respective toxic activity (Fig. 2). Both assay systems produced a near-linear dose response curve. The PF data were plotted as the square root of the area of bluing because this transformation increased the homogeneity of variances and improved the linearity of the response. We observed a highly significant linear regression for both the PF bluing and RIL curves. The curve for PF necro-

sis deviated significantly from linear regression. The data of Fig. 2 were replotted as a regression of each activity on PF units administered (Fig. 3). This was done by applying the definitions of PF and DU given previously to a regression of each activity on the dilution of culture filtrate concentrate administered (not shown). Based on Fig. 3, one DU is equivalent to 1.6 PF units. But as this is expressed on the basis of volume

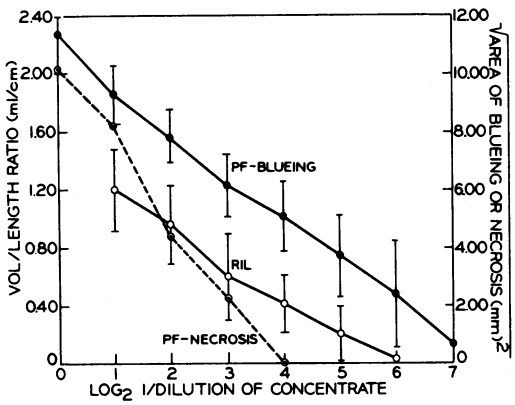

FIG. 2. *PF and RIL activities in cell-free culture filtrates (concentrated six-fold) of B. cereus B-4ac. PF: mean of 15 values ± 1 standard error of the mean. RIL activity: mean of 12 values ± 1 standard error of the mean.*

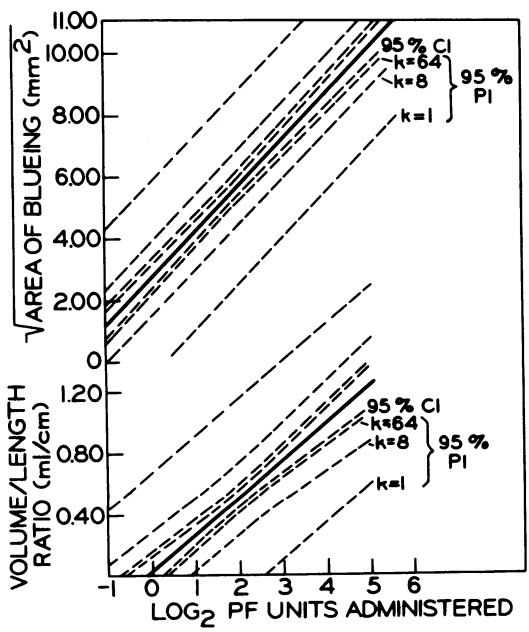

FIG. 3. *Regression of PF bluing (top) and RIL response (bottom) on PF units administered with 95% confidence limits (CL) and prediction limits (PL). Prediction limits based on 1, 8, and 64 replications.*

administered and since the PF assay used only 1/40th the volume of sample used in the RIL assay, the latter, in fact, required 64 times as much toxin to elicit a unit response as did the former.

The confidence and prediction limits established in Fig. 3 demonstrate that a large amount of variability was present in both assays, but the variability of the RIL assay was somewhat greater.

The full implication of this variability is most clearly seen in Fig. 4. This curve predicts (with a 95% probability of being correct) the number of replications that will be required to prove that an observed difference (δ) in toxic activity between two test samples is significant at α = 0.05. Our experience has been that the range of greatest interest is $1.0 \leq \delta \leq 2.0$, and the number of replications most often needed is six to eight. At more than six replications, the relative sensitivity of the PF assay (1.6 δDU/δPF units) is about 1.8 times that of the RIL assay. Consequently, the number of replications in the PF assay is reduced by two from that required in the RIL assay.

Activity of other strains. Once the ability of a single strain to produce a vascular response had been determined, culture filtrates of 10 other strains of *B. cereus* and of representatives of other *Bacillus* species (13 strains) were then tested for permeability-influencing activity. Positive reactions were observed for culture filtrates prepared from 9 of the 10 *B. cereus* strains and for 5 of 5 *B. thuringiensis* strains. The filtrate of the PF-negative strain of *B. cereus* was not active in either ileal loop or guinea pig skin assays. Two of four culture filtrates of *B. mycoides* strains tested gave positive responses in all three assays, and the other two were negative or very weakly positive. Culture filtrates from strains of *B. megaterium*, *B. subtilis*, and *B. licheniformis* displayed none of these activities. It therefore appears that only preparations from *B. cereus* and closely related species are capable of eliciting the characteristic vascular permeability response.

Further characterization of the permeability factor distinguished it from other extracellular substances produced by *B. cereus*.

Relation of PF to phospholipase C and to hemolysin. Thermal stability has been found to be a major differentiating characteristic between phospholipase C, as measured by the lecithovitellin reaction, hemolysin, and both ileal loop fluid inducing activity and dermonecrotic activity (5). Heating was performed by placing 1-ml amounts of culture filtrate in test

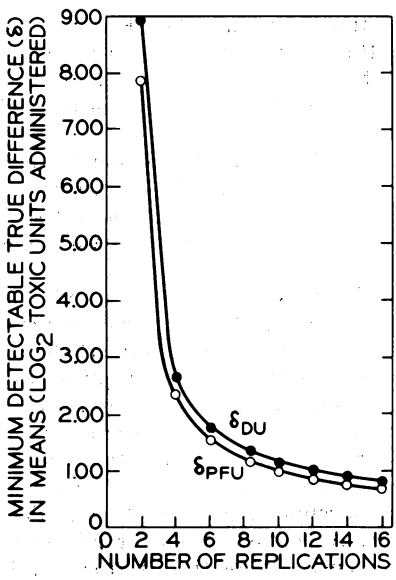

FIG. 4. *Minimum detectable true differences (δ) in means between two test samples as a function of the number of replications performed in PF or RIL assays. The dose is expressed as $\log_2$ toxic units administered (DU or PF units). The curves are established for a 95% probability that the minimum difference will be significant at $\alpha = 0.05$.*

tubes in a constant-temperature water bath for selected periods of time, followed by rapid cooling in an ice bath. Heating of culture filtrate at 56 C for 10 min completely destroyed permeability-influencing activity but had no effect on phospholipase activity. Culture filtrate subjected to 10 min of heat at 56 C produced only the transient increase in vascular permeability characteristic of phospholipase C preparations.

In contrast to the heat stability of phospholipase, *B. cereus* hemolysin has been shown to be quite heat labile (5, 8). In the present study, incubation of culture filtrate at 45 C for 30 min reduced its hemolytic titer by 90% but had no effect on permeability-influencing activity. The stability of PF under these conditions is the same as that of ileal loop and dermonecrotic activities.

Immunological tests also differentiated PF from phospholipase C. Rabbit antiserum was prepared against crude culture filtrate and against purified phospholipase C. The anti-filtrate serum neutralized both phospholipase and permeability-influencing activity of culture filtrate, but anti-phospholipase serum neutralized only phospholipase activity, with no effect on PF.

The single PF-negative strain of *B. cereus* produced normal levels of phospholipase C and hemolysin. Conversely, four strains which possessed PF activity failed to produce phospholipase C. This is a further indication of the non-identity of PF and the other two activities.

Characterization of PF. The relative thermal instability and antigenicity of PF indicated that it was probably protein in nature. In addition, this factor was found to be non-dialyzable when a 5-ml sample of culture filtrate was subjected to dialysis against 1,500 ml of saline for 24 h at 4 C. One hundred percent of permeability-influencing activity was recovered from the dialysand. Addition of ammonium sulfate between 40 and 60% of saturation precipitated PF from BHIG-culture filtrates, with at least 70% recovery of permeability-influencing activity. Further definition of the chemical nature of PF awaits purification of this substance from culture filtrate.

Correlation between ileal loop factor and PF. If the vascular permeability assay is to be used as an alternative measure of enteropathogenicity, the properties of the ileal loop factor and of PF in culture filtrates must be closely correlated. Relative thermal instability, non-dialyzability, and precipitation at 40 to 60% saturation with ammonium sulfate were shared characteristics of both activities. Appearance of these activities during growth of a B-4ac culture was also studied.

A 0.05% inoculum from a 12-h culture was added to 250 ml of BHIG in a 2-liter flask and incubated at 32 C on a gyratory shaker. At selected times after inoculation, the culture was sampled to determine pH, cell numbers (by plate counts on plate count agar), and the presence of ileal loop fluid and permeability-influencing activities in the culture filtrate (Fig.

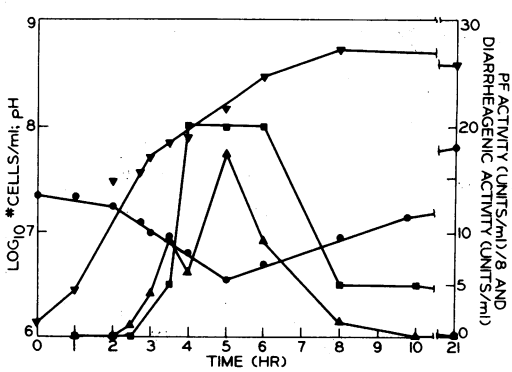

FIG. 5. *Relationship of cell numbers (▼), pH (●), and appearance of PF (■) and diarrheagenic (▲) activities during growth of a culture of B. cereus B-4ac.*

5). The culture began rapid growth with no appreciable lag, and continued for 6 to 7 h. Both ileal loop and permeability-influencing activities appeared in the culture filtrate during exponential-phase growth, at 2.5 and 3 h, respectively. Both activities were at a peak between about 4 and 6 h, the period of time when the pH of the culture reached its lowest point and began to rise again, signaling the induction of the tricarboxylic acid cycle in correlation with the onset of sporulation. Prolonged incubation caused a rapid fall in the levels of both activities, and by 21 h neither was detectable. The cause of this loss of activity is unknown. It is possible that surface denaturation of the responsible protein(s) occurs during continued shaking, or that this protein(s) was sensitive to proteases induced during sporulation.

In addition to the parallel development of ileal loop factor and PF during the growth cycle, the effects of various changes in growth conditions on these two activities were further indication of their relatedness. The choice of growth medium had a significant effect on the measurable activity of PF in culture filtrates. Highest activity was obtained in filtrates derived from BHIG-grown cultures, and somewhat less activity was present in Trypticase soy broth filtrates. PF activity could not be detected in nutrient broth cultures. Similar results were obtained when ileal loop activity was determined for cultures grown in these media (9).

Activities of PF and ileal loop factor in the culture filtrate were also influenced by the extent of aeration. High levels of these activities were measurable in filtrates obtained from shaken cultures, but static cultures, incubated in Erlenmeyer flasks or in filled bottles, produced only barely detectable levels of these factors. The addition of NO_3 as an alternate electron acceptor to these static cultures had no effect on the production of PF and ileal loop factor.

The properties of the permeability-influencing factor described above correlate well with the properties of the ileal loop fluid-inducing factor and of the guinea pig dermal factor (5, 9). Efforts are now underway to isolate these factors from crude culture filtrate. However, until such purification is complete, establishment of these activities as properties of a single entity is not possible. The evidence accumulated so far indicates that the guinea pig dermal assay and now the vascular permeability assay can be used as alternative measures of enterotoxigenicity (in rabbits) of isolates of *B. cereus* and the closely related organisms *B. thuringiensis* and *B. mycoides*. The sensitivity of the vascular permeability assay is about twice that of the ileal loop assay, and, being quicker, cheaper, more reliable, and easier to perform, this assay is well-suited to screening of large numbers of strains for enteropathogenicity or to following the purification of the responsible factor.

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