STUDIES ON IMMUNITY IN ANTHRAX

II. In Vitro Elaboration of Protective Antigen by Non-Proteolytic Mutants of Bacillus anthracis

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Since the work of Bail (1) the edema fluid of cutaneous anthrax lesions has been known to contain an antigen capable of eliciting protective immunity. Sterile extracts of lesions were found to produce active immunity in rabbits, sheep, and guinea pigs, and to a lesser extent in hamsters and mice (2–4). Gladstone (5) demonstrated that the protective antigen was also elaborated during *in vitro* growth of the organism in blood plasma, and that culture filtrates as active as edema fluid could be produced under carefully controlled conditions. It was necessary that the inoculum be small and the incubation time short, otherwise the filtrates were inactive. It was suggested that the destruction of protective antigen which occurred when incubation of cultures was continued beyond 30 hours was due to activity of a protease also elaborated by *Bacillus anthracis* (5). Longer incubation of the culture was permissible and increased yields of the antigen were obtained if the culture was dialyzed against broth in a specialized apparatus during growth (6). The present report describes the isolation and properties of mutants of *B. anthracis* which do not elaborate protease and produce protective antigen during cultivation in media containing plasma.

Materials and Methods

The Vollum strain of *B. anthracis* was used. Spore suspensions were prepared as described previously (7). For the isolation of non-proteolytic mutants a simple method for detection of proteolytic activity of individual colonies was desirable. It was found that addition of 10 per cent brom-cresol purple milk (Difco) to nutrient agar gave a medium of distinct turbidity, and that colonies of *B. anthracis* growing on thin layers of this medium produced, usually within 24 hours, sharply defined zones of clearing, presumably due to hydrolysis of the casein. Use of this medium permitted study of the proteolytic activity of large numbers of colonies, since subculture was unnecessary. Autoclaved milk, cooled to 50°C., was added to melted agar, and the mixture was poured into Petri dishes.

Protective antigen was assayed by active immunization of rabbits, using a modification of the method of Gladstone (5). In order to reduce the time required for the assay an accelerated immunization schedule was used. Five intracutaneous injections of 0.5 ml. each were given at 2-day intervals. 7 days after the last injection of antigen the animals were challenged by

intracutaneous injection of 10,000 spores of the Vollum strain (at least 100 lethal doses) on the back near the posterior axillary line.

EXPERIMENTAL RESULTS

Isolation and Cultural Characteristics of Non-Proteolytic Mutants.—

The stock suspension of spores was diluted in water to a concentration of 8×10^6 spores per ml. and exposed to ultraviolet light in a rotating quartz flask under conditions adjusted to reduce the viable spores to 400 per ml. as indicated by counts on nutrient agar. Appropriate amounts of the irradiated suspension were spread on milk agar plates; these were incubated at 37°C. for 24 hours and examined for non-proteolytic colonies. Five such colonies were observed and subcultured for further study.

On reinoculation onto milk agar all five non-proteolytic cultures produced negligible clearing, but the colony morphology was identical with that of the parent strain. Cultures of the non-proteolytic strains stained by Giemsa technique showed no change in microscopic morphology; no alteration in spore production was evident. Growth of cultures on 50 per cent bovine-serum agar incubated in 20 per cent carbon dioxide was mucoid and identical with that of the parent strain; this indicated that capsule production was unchanged (8). Non-encapsulated mutants could, however, be obtained from the non-proteolytic strains by the method of Sterne (8); these mutants were also non-proteolytic. The non-proteolytic strains did not liquefy nutrient gelatin, whereas the parent strain produced complete liquefaction of this medium. No evidence of reversion to proteolytic activity was noted on subculture, and spore suspensions prepared with the strains yielded only non-proteolytic colonies on milk agar.

The ability of the strains to elaborate a filterable protease in broth cultures was tested. Cultures were grown for 3 days in shallow layers of broth containing 0.5 per cent yeast extract (Difco) and 0.3 per cent beef extract (Difco). Seitz filtrates were tested for proteolytic activity, using casein as a substrate, according to the method of Chow and Peticolas (9).

Filtrates of the parent Vollum cultures were strongly proteolytic, whereas filtrates of mutants were essentially inactive.

Virulence and Rabbit Passage.—The five non-proteolytic strains were tested for virulence by intracutaneous injection of 1000 spores into each of two rabbits. Three of the strains killed both animals, the other two strains, one. Local lesions typical of anthrax developed at the site of injection in all but one of the ten animals. Samples of heart blood were taken shortly before or after death and plated on milk agar. Only non-proteolytic colonies developed. Two strains were carried through two further serial passages in rabbits, and spore suspensions were prepared from the recovered cultures. No proteolytic colonies were observed when these suspensions were plated on milk agar.

The 5 strains were injected respectively into 5 groups of 3 mice, each mouse

receiving 100 spores subcutaneously. Most of the mice died within 3 days, and all of them within 5 days.

Production of Protective Antigen.—The non-proteolytic strains were compared with the parent strain with respect to production of protective antigen in a modification of the synthetic medium of Brewer *et al.* (10), to which 20 per cent sheep plasma was added. Experimental results on production of protectve antigen in this and other media will be presented in a subsequent paper.

The medium was sterilized by filtration through a Seitz filter, and 50 ml. portions were dispensed in Kolle flasks. An inoculum of 10,000 spores was added to each flask, and the cultures were incubated at 37°C. for 16 hours. Good growth occurred with all strains, and each culture was filtered and assayed for protective antigen by active immunization of four rabbits. All but one of the animals survived the challenge.

TABLE I

Influence of Incubation Time on Protective Potency of Filtrates of Vollum and Non-Proteolytic Strains

Experiment	Culture medium	Inoculum strain of *B. anthracis*	Survival ratios* obtained with filtrates of cultures incubated as follows:			
			16 hrs.	22–25 hrs.	40–44 hrs.	72 hrs.
1	Unclotted sheep plasma	Vollum	3/3	3/3	0/3	
		Non-proteolytic	3/3	3/3	3/3	
2	Clotted sheep plasma	Vollum		3/3	1/3	
		Non-proteolytic		3/3	3/3	
3*a*	Unclotted sheep plasma	Vollum		1/3	2/3	0/2
		Non-proteolytic		3/3	1/2	3/3
3*b*	Clotted sheep plasma	Vollum		1/3	0/3	0/3
		Non-proteolytic		3/3	3/3	0/3

* Survival ratios are recorded as the number of immunized rabbits surviving divided by the number of animals challenged. Unimmunized control rabbits invariably died.

This indicates that all of the non-proteolytic strains produced the protective antigen under conditions suitable for antigen production by the parent strains.

To determine whether use of non-proteolytic strains would allow longer incubation of the culture without destruction of the protective antigen, cultures were incubated at 37°C. for various times, and the filtrates tested for protective antigen. Normal sheep plasma was used as culture medium, according to the method of Gladstone (5), except that in some cases the plasma was sterilized by filtration through a Seitz filter; this treatment prevented clotting of the culture medium on addition of calcium chloride.

In each experiment 50 ml. portions of pooled plasma were distributed into Kolle flasks, inoculated with 500 spores per ml. of culture, and sterile calcium chloride solution was added. One non-proteolytic strain was compared with the parent Vollum strain in each experiment. Results of assays of the filtrates for protective antigen are given in Table I.

It will be noted that in each experiment filtrates from cultures of the non-proteolytic strain were active after a longer time of incubation than filtrates of the parent Vollum strain. Some variation may be observed in the protective potencies obtained in the different experiments; this was probably due to the use of different batches of plasma, since a similar variation was observed in other experiments in which several samples of plasma were compared. A single pool of sheep plasma was used in Experiments *3a* and *3b*. The portion used in *3a* was sterilized before inoculation by passage through a Seitz filter, and the portion used in *3b* by passage through a sintered glass filter. With both strains better antigenic activity was obtained with Seitz-filtered plasma.

DISCUSSION

The present evidence that non-proteolytic mutants of *B. anthracis* elaborate the protective antigen under conditions suitable for antigen production by the parent Vollum strain suggests that such mutants should prove valuable for practical *in vitro* production of the antigen. Their superiority will depend upon the extent to which protease activity is the limiting factor in the accumulation of antigen in cultures. Although no direct evidence of destruction of protective antigen by protease has been obtained, the demonstration that cultures of a non-proteolytic strain may be incubated for a considerably longer period than cultures of the proteolytic parent strain without destruction of the antigen suggests that protease does in fact destroy protective antigen. It would seem probable that if the protease also destroys protective antigen *in vivo*, non-proteolytic living vaccines should prove more effective than the living vaccines now in use for animal immunization.

In general, culture filtrates of non-proteolytic strains appeared to be somewhat more potent than culture filtrates of the parent strain, which suggests that greater accumulation of protective antigen does occur in the former. It seems possible that elimination of proteolytic activity will allow simplification of the medium for antigen production, since certain of the complex requirements for antigen production with ordinary strains (11) may be related to suppression of proteolytic activity; experiments of this sort are in progress.

No evidence has been obtained regarding the fundamental nature of the process which led to isolation of the mutants. The change in proteolytic activity appears to be abrupt and stable, and the strains are referred to as mutants in this sense. It is possible that ultraviolet treatment did not cause mutation but aided selection of preexisting mutants; preliminary experiments indicate that some but not all other strains of *B. anthracis* yield non-proteolytic mutants on similar treatment.

The considerable virulence of the mutants for rabbits and mice suggests that protease is not a major factor in virulence of *B. anthracis* under the experimental conditions used. The present data are obviously inadequate to quantify a small change in virulence accompanying loss of proteolytic activity, but they do indicate that any such change is relatively small compared to the almost complete loss of virulence that accompanies loss of the bacterial capsule (8). The inverse relationship between accumulation of the protective antigen of *B. anthracis* and protease activity is apparently similar to the inverse relationship between elaboration of M substance of group A streptococci and activity of the papain-like protease produced by these organisms under certain conditions (12). It would, therefore, seem possible that the phenomenon is of general significance in controlling the accumulation of specific bacterial antigens *in vitro*. If elaboration of other antigens were similarly dependent upon inhibition of specific proteolytic or other enzymes elaborated concurrently in culture, a rational basis would be provided for manipulation of strains or selection of conditions of cultivation for preparation of immunizing antigens of maximum effectiveness.

SUMMARY

Non-proteolytic mutants were isolated from the Vollum strain of *B. anthracis* following ultraviolet treatment. The mutants were virulent for rabbits and mice and their non-proteolytic character was stable in culture and on serial passage in rabbits. The mutants produced protective antigen under suitable conditions of cultivation *in vitro*, and allowed longer incubation of the cultures without destruction of the protective antigen than did the parent strain. The meaning of the results is discussed.

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