Large-Scale Production of Protective Antigen of *Bacillus anthracis* in Anaerobic Cultures[1]

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ABSTRACT

PUZISS, MILTON (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), LEE C. MANNING, JOE W. LYNCH, EUGENE BARCLAY, IRA ABELOW, AND GEORGE G. WRIGHT. Large-scale production of protective antigen of *Bacillus anthracis* in anaerobic cultures. Appl. Microbiol. **11**:330–334. 1963.—A production-proving test was described for the preparation, by the anaerobic culture method, of large volumes of culture filtrate containing immunologically potent protective antigen of *Bacillus anthracis*. The process consisted of the anaerobic culture of a selected production strain in a chemically defined medium. The culture was then clarified and sterilized by filtration through sintered-glass filters. The sterile culture filtrate was adsorbed onto a preformed aluminum hydroxide gel, and the stabilized gel-antigen complex was concentrated. The final product had high immunizing potency, as shown by both in vivo and in vitro assays, and was well tolerated in man. Stability of the product to accelerated aging was good, and storage at 4 C for 1 year caused only a minor loss in protective activity. Large volumes of the highly antigenic gel-adsorbed protective antigen were readily produced by the method described.

The protective antigen elaborated by *Bacillus anthracis* during aerobic growth has been shown to be effective in immunizing animals and man against anthrax (Wright, Green, and Kanode, 1954; Schlingman et al., 1956; Brachman et al., 1962). Subsequent research established that protective antigen was also elaborated in good yield under anaerobic conditions in chemically defined media (Wright, Puziss, and Neely, 1962). Adsorption of the culture filtrate antigen onto a preformed aluminum hydroxide gel, and preservation and stabilization of the adsorbed product, was described by Puziss and Wright (1963). The stable final product had high antigenicity in experimental animals and was well tolerated in man. These studies indicated that the anaerobic process could be readily adapted to production of antigen on a practical scale. The objective of the work presented in this report was a study of the requirements for adaptation of the anaerobic culture method to provide a large-scale process for production of anthrax protective antigen.

[1] In conducting the research reported herein, the investigators adhered to *Principles of Laboratory Animal Care* established by the National Society for Medical Research.

[2] Deceased, March 1962.

MATERIALS AND METHODS

This work was performed primarily at the facilities of the Merck, Sharp and Dohme Division of Merck & Co., Inc., West Point, Pa. All animal immunization and challenge studies were performed at Fort Detrick.

Cultures. Strain V770-NP1-R, a nonencapsulated, nonproteolytic, and avirulent mutant of *B. anthracis* was used as inoculum (Wright et al., 1962). Immunized animals were challenged with the virulent Vollum strain of *B. anthracis* (Puziss and Wright, 1959).

Growth medium. The chemically defined 1095 medium described by Wright et al. (1962) was used; it was made up as a 25-fold concentrated stock and diluted to required volume in a fermentation tank. The chemicals were chemically pure or reagent grade; all water was freshly distilled and obtained from the plant service line at 180 F. Composition of the defined medium is presented in Table 1.

Production equipment. A glass-lined, jacketed, closed tank of 150-gal capacity (special type M; Pfaudler Manufacturing Co., Rochester, N.Y.) served for preparation of medium and growth of the culture. This vessel (Fig. 1) was equipped with a variable-speed impeller for agitation of the contents, and with the customary temperature and motor controls. Cultures were filtered through a combination of sintered-glass clarifier (Chem-Flow Corp., Little Falls, N.J.) and sterilizer units, each 1 ft^2. The clarifier filter (5-μ porosity) was connected in series with two 1-μ porosity sterilizing filters. A flow rate of about 0.82 liter per min was achieved; this required back-flushing of the clarifier filter with sterile water once per hr. A stand-by 5-μ porosity clarifier unit was used during the back-wash period. The back-flushed fluid was collected in a carboy, autoclaved, and discarded. The filtration arrangement is shown in Fig. 2.

Sterile culture filtrate was passed into a second, closed, glass-lined, jacketed tank (E.S. series; Pfaudler Manufacturing Co., Rochester, N.Y.) for adsorption and holding

at 4 C. This tank was also equipped with an impeller and the customary refrigerant and motor controls. The gel-antigen complex was processed in an International centrifuge (model 13L; International Equipment Co., Boston, Mass.).

Aluminum hydroxide gel. The gel used as adsorbent and adjuvant for the antigen was prepared by the method of Hansen (1953) in a glass-lined, steam-jacketed, open tank equipped with stirring paddles. A continuous-flow nozzle-discharge centrifuge (Sharples Corp., Philadelphia, Pa.) was used to remove and wash the gel. The final gel product was tested for its antigen-adsorbing ability with a culture filtrate of known immunizing activity. Standard gel of proved antigen-adsorbing capacity, kindly supplied by Inga Scheibel, Statens Seruminstitut, Copenhagen, Denmark, was used as an adsorbent control (Puziss and Wright, 1963). The gel product used in the present work resembled the standard Danish gel in its adsorptive capacity for antigen.

Assay methods. Culture filtrates were tested for in vitro antigen activity by the complement-fixation method of McGann, Stearman, and Wright (1961), and also by the agar gel diffusion method of Thorne and Belton (1957). In vivo-immunizing activity was assayed by immunization and challenge of rabbits (Puziss and Wright, 1963). All animals received a single 0.5-ml subcutaneous immunizing dose of antigen diluted in saline. Then, 2 weeks later the animals and unimmunized controls were challenged intracutaneously with 10,000 spores of the challenge strain in a 0.25-ml volume. This dose represented 500 to 1,000 LD_{50}. Residual glucose in the culture was determined by the anthrone method of Morris, as described by Neish (1952). The bacterial counts were estimated turbidimetrically in a colorimeter against a nephelometric standard suspension of *Salmonella typhosa* obtained from the Division of Biologics Standards, National Institutes of Health, Bethesda, Md. Aluminum, aluminum oxide concentration, and formalin content in the final antigen product were determined by the methods described in the USP. Sterility and safety tests performed during production, as well as on the final product, were in accordance with Public Health Service Regulations (1961).

Process description of medium. AB-G and C stocks (12

TABLE 1. *Constituents requred for 300 liters of 1095 medium*

Constituent*	Concn
AB-G stock	*g/12 liters*
Biotin, crystalline	0.15
Thiamine·HCl	0.12
Adenosine	0.30
L-Alanine	2.70
L-Tryptophan	3.12
DL-Serine	6.24
L-Arginine·HCl	6.24
L-Proline	8.76
Glycine	8.76
DL-Methionine	9.00
DL-Threonine (allo-free)	18.00
DL-Valine	18.00
DL-Aspartic acid	19.20
DL-Isoleucine	19.20
DL-Phenylalanine	20.40
L-Histidine·HCl	28.80
DL-Leucine	38.40
L-Glutamic acid	50.40
$MnSO_4 \cdot H_2O$	1.20
$MgSO_4 \cdot 7H_2O$	3.00
$CaCl_2 \cdot 2H_2O$	4.44
Glucose	300
Guanine·HCl†	2.76

* Included in 1095 medium were the solutions listed below. C stock solution contained (g/12 liters): KH_2PO_4, 204; and K_2HPO_4, 261. Ferrous sulfate solution, which was heated to dissolve and sterilized by filtration, contained: $FeSO_4 \cdot 7H_2O$, 0.87 g/3 liters; and concentrated HCl, 4.5 ml/3 liters. Pyridoxal solution, which was sterilized by filtration, contained 0.3 g/600 ml of pyridoxal·HCl. Sodium bicarbonate solution, sterilized by autoclaving for 45 min at 121 C, contained 750 g/6 liters of $NaHCO_3$.

† Boiled to dissolve in 600 ml of water plus 9.0 ml of concentrated HCl, added to AB stock, and brought to a final volume of 12 liters.

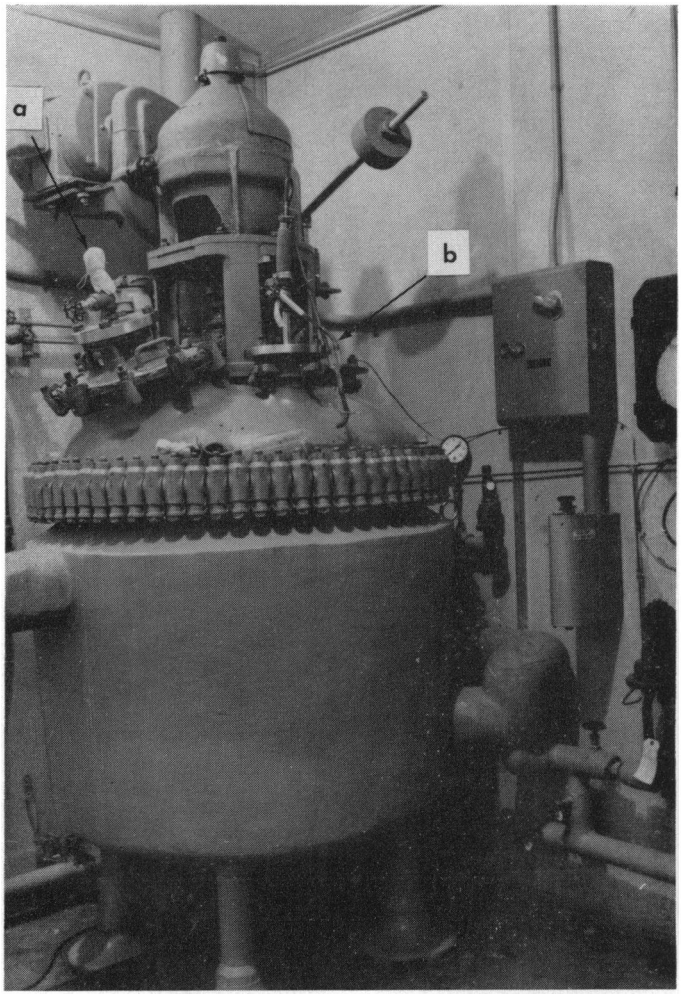

FIG. 1. *Steam-jacketed fermentation tank and controls, showing the inoculating port (a), and the sampling tube (b).*

liters each) were added to 266.3 liters of distilled water contained in a 150-gal fermentation tank. The tank and its contents were sterilized by heating to 120 C and cooling immediately to 37 C. After the temperature reached 100 C, pressure was equilibrated by allowing air to enter through a sterile air filter; 6 liters of sterile sodium bicarbonate (12%) were then added. Sterile ferrous sulfate and pyridoxal hydrochloride solutions, pooled in a 20-liter Pyrex glass bottle, were added to the contents of the tank, and the tank was inoculated with the seed culture. Final pH of the medium was about 7.9. A sterile tube, inserted through the inoculating valve, was used to introduce nitrogen (sterilized by a filter) into the tank to a pressure of 5 psi. The gas was introduced close to the surface of the medium for 25 min, and displaced the air through the top valve of the tank; the valve was then closed.

The culture was incubated at 36 to 38 C under nitrogen for approximately 27 hr, with agitation at a constant rate of 20 rev/min. Samples for the various tests were removed through a sterile tube at selected intervals during growth.

Filtration and adsorption. At the termination of incubation, the pH of the culture, which had dropped to pH 6.9, was raised to pH 8.0 with sterile 2 N sodium hydroxide and the culture cooled to 18 to 20 C. A pressure of about 10 psi of nitrogen was applied, and the culture was forced through a glass valve in the bottom of the tank, through the clarifying and sterilizing filters, and into the holding tank. Approximately 6 hr were required to filter the 300 liters of culture.

Sterile aluminum hydroxide gel (12 liters) at a temperature of 22 C was added to the filtrate. The mixture was agitated for 30 sec at 60 rev/min and then cooled to 4 C. Three times daily for 5 days, the suspension was agitated for 30 sec. An alternate method, preferable when proper equipment is available, would be to agitate continuously at 15 to 20 rev/min for 2 days (Puziss and Wright, 1963). Agitation was discontinued 20 hr prior to removal of adsorbed antigen from the tank to allow the material to settle. Approximately 31 liters of the concentrated suspension of adsorbed antigen were removed through the bottom valve. The supernatant was discarded.

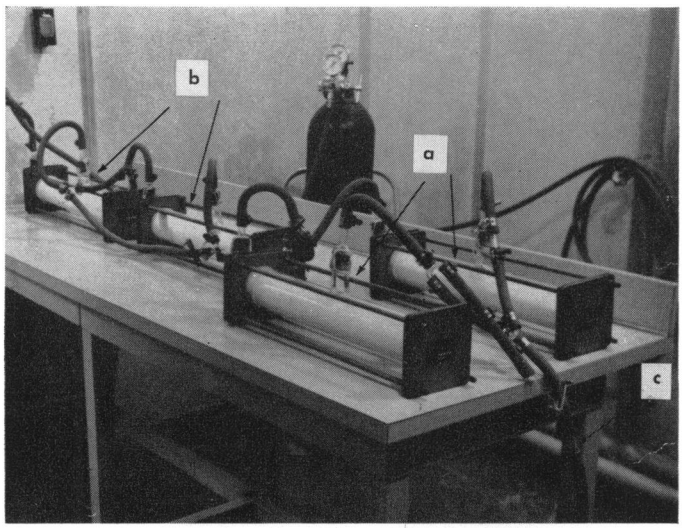

FIG. 2. *Chem-Flow filter assembly. The clarifier filters (a) are attached to the sterilizer filters (b) arranged in series. Culture from the fermentor enters the assembly from the tube at right (c); sterile filtrate leaves in the tube at left through an opening in the wall.*

Final processing. The adsorbed antigen was distributed aseptically into sterile 1.5-liter Pyrex centrifuge bottles, each containing several sterile glass beads. A centrifugal force of 780 × *g* was applied for 10 min at 4 C, and the supernatant was removed aseptically by vacuum from each bottle and discarded. The gel slurry remaining was strained into a sterile 45-liter Pyrex carboy through a sterile #62 mesh nylon strainer. The pooled slurry (approximately 10 liters) was resuspended in cold, sterile, physiological saline solution to 28.5 liters. Preservatives were added and the carboy was held at 4 C until potency and safety tests were completed. The preservative consisted of 750 ml of a 1:1,000 solution of recrystallized benzethonium chloride (Phemerol; Parke, Davis & Co., Detroit, Mich.) to a 1:40,000 final concentration. As a stabilizer, 750 ml of 1% formalin were added to a final concentration of 0.0092% formaldehyde. The final volume of product was 30 liters; this represented a tenfold concentration from the original 300 liters of culture filtrate. Determinations of sterility by cultural tests, safety tests with mice and guinea pigs, tests for antigenicity in rabbits, and tests for aluminum and formalin content were performed. Finally, vials were filled with 20-ml quantities of the antigen. Approximately 60,000 doses were obtained from the 300-liter lot.

RESULTS

In the early phases of the investigation, the fermentation tank containing the culture medium was inoculated with 500 spores/ml of the production strain of *B. anthracis*, as described previously (Puziss and Wright, 1963). Under these conditions, the initiation of growth was delayed, maximal turbidity and utilization of glucose were reduced, and only small amounts of protective antigen were detected in culture filtrates.

The difficulty was overcome by seeding the tank with an actively growing vegetative inoculum rather than with the spore suspension. The culture medium (10 liters) in a carboy was inoculated with 10^7 spores of strain V770-NP1-R, incubated statically in air for 26 hr at 37 C, and added to the main lot of medium. Under these conditions, good growth and maximal accumulation of antigen occurred in the 300-liter culture after incubation for approximately 26 hr. At intervals during the incubation period, determinations of the pH, the bacterial count, the complement-fixation titer, and the residual glucose were carried out; data compiled from several production experiments are shown in Fig. 3. Glucose utilization was the most sensitive and useful indicator of the course of the fermentation; earlier studies had shown that maximal antigen

accumulation coincided with the point at which glucose in the medium approached exhaustion (Puziss and Wright, 1959). After 18 to 20 hr of growth, it was possible to extrapolate the glucose-utilization curve to estimate the time at which antigen elaboration would be at a maximum and filtration of culture could be initiated. Antigenicity of five consecutive production lots as measured by in vivo and in vitro assays is presented in Table 2.

Stability to accelerated aging of the gel-adsorbed product at 37 C was studied. Antigen 9 was incubated at 37 C and assayed in rabbits at weekly intervals to determine the loss of immunogenic potency. The results indicated that the antigen had considerable stability to accelerated aging; approximately 60% of the test animals survived challenge after immunization with antigen heated at 37 C for 8 weeks (Fig. 4). Stability to accelerated aging of the product was equivalent to that of an experimental gel-adsorbed antigen prepared previously in the laboratory (Puziss and Wright, 1963). Several lots of antigen were reassayed after storage for 1 year at 4 C. Results of these assays indicated that antigenicity of the stored product decreased to a slight but minor extent in some of the lots (Table 3).

Several lots of gel-adsorbed antigen have been used in a continuing program of immunization of personnel occupationally exposed to anthrax, either in research laboratories or in industry. The antigen was well tolerated; mild and transitory local reactions were elicited at the site of injection in a very small percentage of those immunized. Further studies on the antigenicity of the product are in progress.

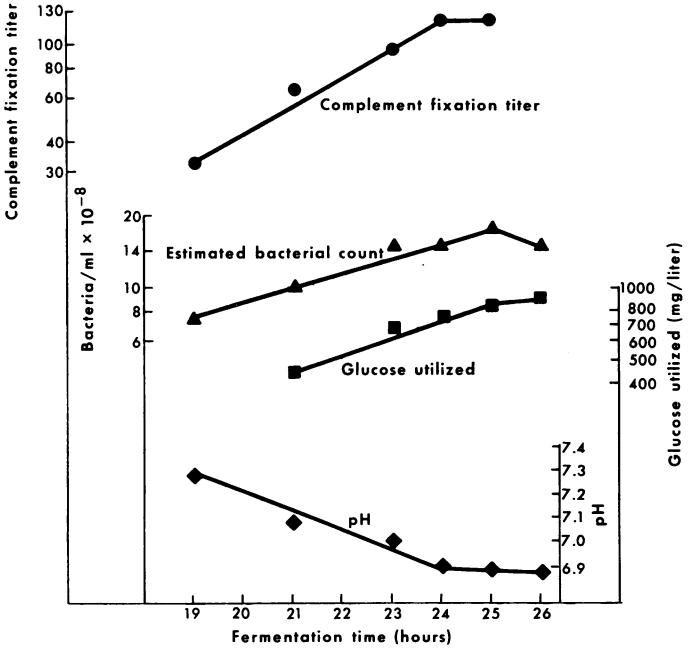

FIG. 3. *Relationship of complement-fixation titer, bacterial count, glucose utilization, and pH change in cultures of Bacillus anthracis.*

TABLE 2. *Antigenicity obtained in six successive 300-liter cultures*

Lot no.	Titers of culture filtrate		Antigenicity of gel-adsorbed product*		
	Complement fixation (50% units/ml)	Gel diffusion end point	Antigen dilution 1:10	1:30	1:90
5	160	1:4	4/8; 6/8		
6	160	1:4	6/8		
7	160	1:4	4/8; 4/8		
8	160	1:2	7/8; 7/8	5/8	5/8
9	160	1:2	7/8; 8/8	7/8	7/8
10	160	1:2	6/8		

* The antigen product was diluted with saline as shown; a single 0.5-ml immunizing injection was given. Results show the number of surviving rabbits over the total number challenged. Two control animals were used in each experiment, and none survived.

Discussion

The study achieved its objective, the development of a laboratory method into a process suitable for large-scale

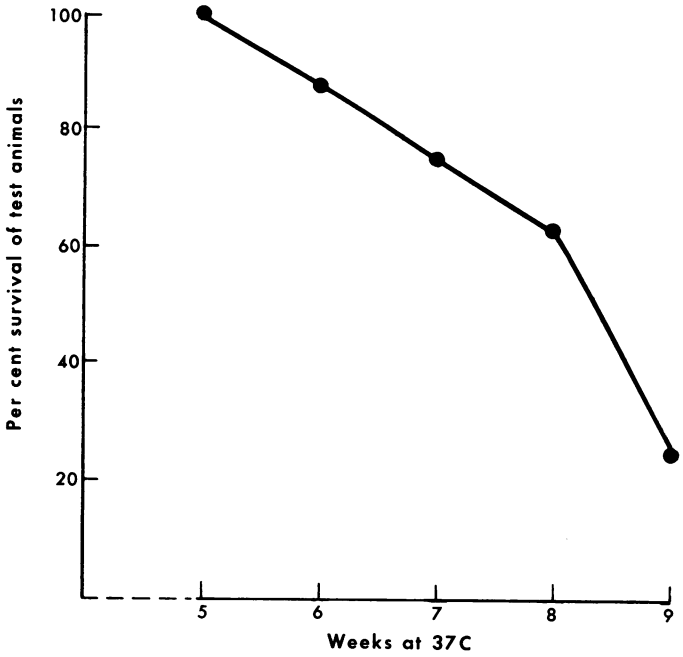

FIG. 4. *Stability of the gel-adsorbed product after heating at 37 C.*

TABLE 3. *Antigenicity of gel-adsorbed antigen after storage at 4 C for 1 year*

Lot no.	Survival ratio*		
	Antigen dilution 1:10	1:30	1:90
8	5/6	—	3/6
9	5/6	4/6	3/6
10	8/8	—	6/8

* The antigen product was diluted with saline as shown; a single 0.5-ml immunizing injection was given. Results show the number of surviving rabbits over the total number challenged. Two control animals were used in each experiment, and none survived.

production of the protective antigen of *B. anthracis*. The process described herein appears to be a reliable method for producing large volumes of the antigen in a form suitable for immunization of man; it is adaptable to a further increase in the scale of production should the need exist. Replacement of the spore inoculum with an actively growing vegetative inoculum was the only change that was required to adapt the laboratory procedure to use with 300-liter lots of culture in tanks. Substitution of the vegetative inoculum not only led to satisfactory growth and elaboration of antigen, but also reduced the incubation time of the tank culture to approximately 26 hr. No explanation was established for failure of the tank-scale cultures to grow as satisfactorily as the laboratory-scale cultures when a spore inoculum was used.

The preparations of antigen produced in the present study appear to be at least equivalent to those prepared in the laboratory in all respects tested. Complement-fixation titers of the culture filtrates and the antigenicity of the adsorbed products in rabbits were generally similar (Puziss and Wright, 1963). Gel diffusion titers of the present filtrates were not significantly different from unpublished titers obtained with laboratory lots of filtrate; no valid comparison can be made with diffusion titers recorded by others because of the use of different antisera and the probable influence of minor variations in technique. The present preparations also resembled laboratory preparations with respect to stability during storage at 4 C and during accelerated aging at 37 C.

The least satisfactory portion of the over-all process was the centrifugation of the gel-adsorbed product. This procedure is time-consuming and affords a potential source of contamination of the product. Newer concepts in the production method are under consideration in an effort to eliminate the troublesome centrifugation and maintain an essentially closed production system.

The protective antigen is a rather labile substance elaborated only under carefully controlled conditions. The satisfactory results obtained in production of this substance in 300-liter lots may justify attempts to produce other labile biologicals on a large scale by bioengineering techniques.

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