Nonproteolytic, Avirulent *Bacillus anthracis* as a Live Vaccine

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ABSTRACT

FUBRA, ERNEST S. (Federal Department of Veterinary Research, Vom, Nigeria). Nonproteolytic, avirulent *Bacillus anthracis* as a live vaccine. J. Bacteriol. 91:930-933. 1966.—A nonproteolytic mutant, derived from the Sterne strain of *Bacillus anthracis* by exposure to ultraviolet radiation, was used for vaccination of guinea pigs. Vaccine prepared from the parent Sterne strain was used in comparable immunization procedure. Comparison of the protection induced by the two vaccines showed that use of the nonproteolytic mutant was from 10 to 1,000 times as effective as the Sterne strain.

Anthrax spore vaccine prepared from the Sterne strain of *Bacillus anthracis* is widely used in many countries. Immunity conferred by such vaccine has been shown to be due to the elaboration of a protective antigen. This antigen has been extensively studied (1, 2, 4, 5, 7, 9-14). Gladstone (4) found that the protective antigen elaborated in vitro is destroyed on prolonged incubation of the culture. Such loss in protective antigen is not experienced with a nonproteolytic mutant of *B. anthracis* (11).

This paper presents results of immunization of guinea pigs with a nonproteolytic mutant of the Sterne strain of *B. anthracis* (live spore vaccine). Its immunizing efficiency was also compared with that of a spore vaccine prepared from the Sterne strain of *B. anthracis* on the basis of the immunity index (3).

MATERIALS AND METHODS

Animals. Guinea pigs used for the immunization experiments were bred and raised at the Federal Department of Veterinary Research, Vom, Nigeria. Animals weighing approximately 350 g each were selected; each group of 10 animals was kept in a separate cage.

Bacterial cultures. The Sterne strain of *B. anthracis* (34F₂) was used for some of the immunization experiments and also for the derivation of the nonproteolytic mutant. Stock cultures of *B. anthracis* 34F₂ were stored as ampoules of freeze-dried spore suspensions, in a freezer.

The attenuated Pasteur II strain of *B. anthracis*, maintained as a saline-spore suspension in 50%

glycerol, was used for challenge of immunized and control animals. The LD₅₀ values for guinea pigs were determined immediately prior to challenge, since the test animals were known to show seasonal variation in resistance (*unpublished data*).

Media. Nutrient agar and blood-agar were used for all bacterial cultivation. For the isolation of nonproteolytic colonies, nutrient agar plus 10% milk and 2.5% bromocresol purple, was poured in thin plates.

Preparation of spore suspension. An ampoule of freeze-dried spores of *B. anthracis* was reconstituted with sterile physiological saline, streaked on blood-agar plates, and incubated at 37 C for 24 hr. Isolated colonies were transferred to sterile physiological saline. This suspension was then used for the inoculation of nutrient agar in Roux flasks. Incubation of seeded Roux flasks was at 37 C, and was continued (usually for 2 or 3 days) until representative samples from the flasks indicated at least 90% sporulation. Smears from the samples were stained by the Malachite green-safranin method, and the average proportion of spores to vegetative cells was determined from five random fields. The contents of each flask were harvested by the addition of 15 ml of sterile physiological saline containing glass beads. Pooled suspensions were heated in a water bath at 60 C for 30 min and were allowed to cool before samples were removed for determination of the number of viable spores. The suspensions were standardized so that each contained 2×10^8 spores per milliliter.

Induction and isolation of the nonproteolytic mutant. Five-milliliter amounts of saline spore suspension containing approximately 10^7 spores per milliliter were irradiated in 9-cm petri dishes for 45 min at 20 cm from the filament of a 30-w bactericidal ultraviolet unit (model 1497, K. Hanovia Lamps, Slough, England). After irradiation, approximately 400 viable spores were left per milliliter of suspension.

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TABLE 1. Comparative protection^a induced in guinea pigs by the Sterne strain of *Bacillus anthracis* and the nonproteolytic *B. anthracis* mutant

Immunization treatment	Trial 1			Trial 2			Trial 3			Trial 4			Mean immunity index $\pm$ se	Avg proportion of survivors
	H ^b	I ^c	S/N ^d	H	I	S/N	H	I	S/N	H	I	S/N		
Unvaccinated controls	81.97	0	0/10	71.94	0	0/10	53.19	0	0/10	53.19	0	0/10	0	0/10
Standard Sterne strain vaccine														
2 $\times$ 10 ⁶ spores	118.06	0.67	3/10	138.12	1.59	3/10	88.49	1.79	1/10	94.34	1.95	2/10	1.50 $\pm$ 0.29	2.25/10
2 $\times$ 10 ⁷ spores	205.76	1.75	6/10	S ^e	NC	10/10	90.09	1.83	0/10	111.48	2.34	0/10	1.97 $\pm$ 0.18	4/10
2 $\times$ 10 ⁸ spores	S	NC ^f	10/10	S	NC	10/10	124.06	2.56	3/10	151.06	2.90	2/10	2.73 $\pm$ 0.17	6.25/10
Mutant vaccine														
2 $\times$ 10 ⁴ spores	—	—	—	—	—	—	77.51	1.41	0/10	78.74	1.51	0/10	1.46 $\pm$ 0.16	0/10
2 $\times$ 10 ⁶ spores	—	—	—	261.78	2.40	6/10	151.52	2.90	5/10	154.79	2.94	5/10	2.75 $\pm$ 0.17	5.33/10
2 $\times$ 10 ⁶ spores	357.14	2.24	7/10	369.0	2.70	7/10	306.75	3.69	7/10	308.64	3.71	7/10	3.11 $\pm$ 0.36	7/10
2 $\times$ 10 ⁷ spores	386.1	2.29	7/10	S	NC	10/10	—	—	—	—	—	—	—	8.5/10
2 $\times$ 10 ⁸ spores	S	NC	10/10	—	—	—	—	—	—	—	—	—	—	10/10

^a Against challenge with approximately 100 LD₅₀ of the Pasteur II strain of *B. anthracis*, 21 days after vaccination.
^b Harmonic mean time-to-death (H) is derived from the formula: $1/H = (1/N) (1/X)$, where N = number of animals per group, X = time-to-death (hours) after challenge of an individual within the group.
^c Immunity index (I) is defined in text.
^d Number of animals surviving at 7 days after challenge/total number of animals in each group.
^e All animals in the group were still alive 7 days after challenge.
^f Not calculated.

The suspension was streaked on each of several thin plates of bromocresol purple-milk-agar and incubated at 37 C for 24 hr. Colonies showing no zones of proteolysis were used for the inoculation of fresh bromocresol purple-milk-agar plates, to confirm that loss of proteolytic activity was stable. A single nonproteolytic colony was then used for the preparation of spore suspensions as described above.

Immunization procedure. Selected guinea pigs were caged in groups of ten and observed for 1 week before immunization was commenced. Animals in a given cage received the same immunizing dose of spores. For the "standard" vaccine, the dose ranged from 2×10^6 to 2×10^8 spores in 1 ml of physiological saline, given subcutaneously in the right thigh; for the "nonproteolytic mutant vaccine," the dose levels were from 2×10^4 to 2×10^8 spores, injected subcutaneously in the right thigh in 1-ml quantities. The former represents the protocol used at Vom for the testing of anthrax spore vaccine. The latter dose range was fixed from preliminary experiments. One group of guinea pigs was used as an unvaccinated control for both types of vaccine being tested. All animals were challenged 21 days later with approximately 100 LD₅₀ of the Pasteur II strain *B. anthracis* spores in 1 ml, administered subcutaneously in the left thigh. Challenged animals were observed for 1 week, during which time-to-death for each animal was recorded (in hours). That death was due to anthrax was confirmed at postmortem examination (i.e., presence of characteristic gelatinous edema; enlarged heart and spleen; recovery of typical, capsulated *B. anthracis* from the heart blood, spleen, and site of challenge, as shown by polychrome-methylene blue staining; and isolation from heart blood and spleen of typical *B. anthracis* colonies on nutrient agar and blood-agar). From the accumulated data, an immunity index (3) was calculated.

RESULTS

The comparative results of protection against challenge with 100 (guinea pig) LD₅₀ of Pasteur II strain *B. anthracis*, after vaccination with the Sterne strain of *B. anthracis* and the nonproteolytic mutant, are shown in Table 1. The nonproteolytic mutant gave better protection than the standard spore vaccine strain. This difference ranged from 10-fold to approximately 1,000-fold; 2×10^8 spores of the mutant gave protection comparable to that conferred by 2×10^8 spores of the Sterne strain (immunity index of 2.9 and 2.94, respectively). The immunity index is computed from the formula (3):

$$\text{immunity index (I)} = 100/b(t_i - t_o)/(t_i t_o)$$

where b = linear slope of dose-response curve (0.42); t_o = harmonic mean time-to-death (hours) of control animals; and t_i = harmonic mean time-to-death of immunized animals.

The average proportion of survivors to total number of challenged animals per group, though

a less absolute indication of protective efficiency, also brings out the superiority of the mutant over the parent strain. Such proportions ranged from 2.25/10 (22.5%) in the group receiving 2×10^6 spores, to 6.25/10 (62.5%) for animals given 2×10^8 spores of the parent Sterne strain, whereas values for animals immunized with the mutant ranged from 5.3/10 (53%) for the group injected with 2×10^6 spores, to 8.5/10 (85%) for those receiving 2×10^7 spores (Table 1).

DISCUSSION

Gladstone (4), using the Weybridge strain of *B. anthracis*, noted that there was always loss of protective antigen on prolonged incubation in vitro (i.e., after 18 hr at 37 C). He also thought that such destruction might occur in vivo but suggested that "the rate of production and the amount produced might well more than compensate for any destruction that occurred." That loss of protective antigen does occur in vivo may be inferred from the difference in protective efficiency of the proteolytic strain and the nonproteolytic mutant of the same strain. Further, compensation by the animal body may very well be overshadowed in time by the production of a proteolytic enzyme, in which case the peak of protective antigen production and therefore strongest protection may be before the 21-day period allowed before challenge. However, the difference in protective efficiency may be due to a number of factors and should not be entirely attributed to the nonproteolytic character of the mutant.

The results indicate that the mutant, used as a live-spore vaccine, may be much more effective than the widely used spore vaccine prepared from the Sterne strain. Derivation of the mutant from the Sterne strain does not entail elaborate techniques, and only one induction is necessary since no reversion to the proteolytic parent type occurs. There is no apparent morphological difference between the parent strain and the mutant. Further, both sporulate easily and to an adequate extent on plain nutrient agar.

It may be necessary to determine whether the duration of protection is altered by use of the mutant, although there is no evidence at this time to suggest that this factor would be adversely affected.

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