Development of Antibodies to Protective Antigen and Lethal Factor Components of Anthrax Toxin in Humans and Guinea Pigs and Their Relevance to Protective Immunity

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A competitive inhibition enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies in serum to the protective antigen (PA) and lethal factor (LF) components of anthrax toxin. Current human vaccination schedules with an acellular vaccine induce predictable and lasting antibody titers to PA and, when present in the vaccine, to LF. Live spore vaccines administered to guinea pigs in a single dose conferred significantly better protection than the human vaccines (P<0.001), although they elicited significantly lower (P<0.0005) anti-PA and anti-LF titers at the time of challenge with virulent *Bacillus anthracis*. Substantial anti-PA and anti-LF titers may not, therefore, indicate solid protective immunity against anthrax infection. The ELISA system was also shown to be capable of detecting anti-PA and anti-LF antibodies in the sera of individuals with histories of clinical anthrax. The advantage of ELISA over the Ouchterlony gel diffusion test and indirect microhemagglutination assay are demonstrated. There was a highly significant degree of correlation between ELISA and the indirect microhemagglutination assay (P<0.0005); but ELISA was markedly superior in terms of reproducibility, reliability, specificity, speed, and simplicity in performance and stability of the bound antigen.

The heat-attenuated vaccines against anthrax based on the original anthrax vaccine of Pasteur (18) suffered from the problems of declining potencies (25) and variations in virulence; as a result of this, they could not be administered safely to certain species (22). An improved vaccine based on an avirulent noncapsulated variant of *Bacillus anthracis* was developed by Sterne (23-25), and the Sterne strain (24) remains the active component of major veterinary live spore vaccines today.

Elucidation of the nature of the three-component anthrax toxin and its relevance to pathogenesis and immunity (10, 14) led to the development in the 1950s of nonliving or chemical vaccines for human use. The British vaccine (1, 6) consists of alum-precipitated toxin protein (3) elaborated by the Sterne strain when grown in the supplemented Casamino Acids medium of Thorne and Belton (27). The U.S. vaccine consists of antigen produced anaerobically in chemically defined medium 1095 (19, 33) and adsorbed onto aluminum hydroxide gel (20). The recommended doses and schedules were based on the animal immunization protocols used to demonstrate protection during vaccine development (4, 6).

One field study (4) indicated that the chemical vaccines only conferred partial immunity on persons with occupational risk, and reservations that were expressed in the 1960s (14) on the probable efficacy of these vaccines persist today. It is apparent that a more effective second-generation vaccine for humans is needed.

A major obstacle to improved anthrax vaccine research has been the lack of a convenient and sensitive method of monitoring the antibody responses they induce. For over two decades, anthrax serology has depended on the Ouchterlony gel diffusion test (21) which, in turn, replaced earlier, in vivo passive protection (3) and edema neutralization (2) tests.

The Ouchterlony test is, however, too insensitive for direct detection of antibodies in the sera of vaccinated individuals. Its use for this purpose requires back titration of standard antigen, preincubated with the test serum, against standard antiserum from an animal hyperimmunized with repeated injections of live spore vaccine.

Although still in regular use, the Ouchterlony test is insufficiently sensitive and discriminating for detailed analysis of antibody responses in vaccinees or for reliable confirmation of natural infections. In addition, it is usually performed with standard antisera raised to live spore vaccine and is *sensu strictu*, nonspecific.

Improved methods of purifying the protective antigen (PA) component of anthrax toxin (9, 30) led to the application of indirect microhemagglutination (IMHA; alias passive hemagglutination) for detecting anti-PA antibodies (5). This was markedly more sensitive and specific than gel diffusion and became the test of choice in the United States. IMHA suffers, however, from poor reproducibility, batch-to-batch variability in sheep erythrocytes, and instability of sensitized erythrocyte-antigen preparations (11).

The enzyme-linked immunosorbent assay (ELISA) for detection of antibody to anthrax toxin components is reproducible, rapid, and easy to prepare (11); in addition, the antigen-coated plastic plates have a shelf life of several weeks. Various versions of the ELISA are in use in different laboratories; we report here the results obtained with a competitive inhibition ELISA used to measure antibody responses to PA and lethal factor (LF), a second anthrax toxin component, in vaccinated humans and guinea pigs. The potential value of this EL1SA for confirmation of true anthrax infections is also demonstrated.

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MATERIALS AND METHODS

Antigens. The PA used in this study was prepared, in part, in the Vaccine Research and Production Laboratory and, in part, kindly supplied by S. H. Leppla, U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Md. In the Vaccine Research and Production Laboratory, following a modified version of the method of Leppla (13), cell-free filtrates of Sterne strain cultures in the supplemented
Casamino Acids (Difco Laboratories, Detroit, Mich.) medium of Thorne and Belton (27) were concentrated and partially purified by ammonium sulfate precipitation (70% saturation), equilibrated with 10mM Tris buffer containing 2 mM EDTA and 2 mM mercaptoethanol, and loaded on hydroxylapatite

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columns. Elution with 0.075 M potassium
phosphate buffer (pH 7.2) yielded PA at a high degree of purity, as assessed by polyacrylamide gel electrophoresis.

The LF used in this study was also supplied by S. H. Leppla.

Human vaccinee sera. The prescribed schedule for human vaccination against anthrax consists of a short course of three intramuscular injections (0.5 ml of vaccine) at 3-week intervals, followed by a fourth dose at 6 months and annual booster doses. Sera from 10 individuals. not previously vaccinated, were obtained just before each injection and 10 to 20 days after the third and fourth doses of British vaccine (product license 1511/0037, prepared for the Department of Health and Social Security in the Vaccine Research and Production Laboratory). In five of the vaccinees, the prescribed schedule was adhered to reasonably closely; in three others, there was a delay of 6 months between doses 2 and 3. In the remaining two individuals, the initial short course was reduced to half the recommended dose injected at 10-day intervals to assess the difference in response to smaller doses administered over shorter time intervals. Sera from a further six British vaccinees after the administration of dose 3 were also examined.

In addition, sera from 31 persons collected at a single session for reasons unrelated to anthrax vaccination were tested. These persons had all completed a full course of anthrax vaccination with the British vaccine at some time in the past, including at least one annual boaster.

Finally, sera from 30 U.S. personnel collected 2 weeks after completion of the short course of vaccination with the U.S. vaccine (U.S. license no. 99, prepared by the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich.) were also examined. These sera were donated by J. Ezzell, Division of Bacteriology, U.S. Army Medical Research Institute of Infectious Disease).

Human sera. A total of 77 sera collected from patients with clinically diagnosed anthrax in the Matabeleland district of Zimbabwe, where anthrax is endemic (7, 28), were examined for anti-PA antibody. A total of 15 serum samples, 10 positive for anti-PA antibody and 5 negative, were also tested for anti-LF antibody.

Guinea pig sera. Guinea pigs were randomly selected from large groups which had been vaccinated prior to challenge. Eight animals were bled before vaccination as preinoculation controls. Two groups were inoculated subcutaneously with standard human doses (0.5 ml) of the British and U.S. human-type vaccines on days 0, 14, and 28. Eight animals drawn from each of these groups were bled before dose 3 on day 28, and an additional eight animals were bled 2 weeks later, i.e., just before challenge.

Two more groups were vaccinated with live spore Sterne strain British animal vaccine (Wellcome Laboratories, Beckenham, Kent, United Kingdom) and with a vaccine derived from Russian STI live spore vaccine (Tobol'sk Bioplant, Ministry of Agriculture, Moscow, USSR). Each vaccine was administered as a single injection of 0.2 ml containing the specified dose of not more than 5 x 10^6 spores in the case of the British vaccine and an estimated 5 x 10^5 spores in the case of the STI vaccine. Eight animals from each group were selected at random and bled at 3 weeks, and an additional eight animals were bled just before challenge at 6 weeks postinoculation.

A proportion of vaccinated animals in all groups succumbed to challenge. In the case of those vaccinated with the live spore vaccines, survivors were held for rechallenge after a further 3 weeks, and eight of these animals were bled for antibody tests.

ELISA procedure. The ELISA procedures used in this study were essentially those of Williams et al. (31, 32). Each of the 96 wells in a flat-bottom microelisa plate (Dynatech Laboratories, Inc., Alexandria, Va.) was sensitized with PA or LF by the addition of 75 µl of a solution containing an estimated 15 µg of the antigen per ml in coating buffer (0.16 g of

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Na_2CO_3, 0.29 g of $NaHCO_3$, 100 ml of distilled water [pH 9.6]). The plates were allowed to dry completely at 37°C; unbound sites were blocked with bovine serum albumin (150 µl of a 4% solution in distilled water was added to each well and incubated for 30 min at 37°C). After extensive washing, the plates were allowed to dry; at this stage they could be stored for several weeks without risk of deterioration.

In preparation for testing, 50 µl of diluent phosphate-buffered saline with 0.05% Tween 20 was added to all the wells of odd-numbered rows on the sensitized microelisa plate, and a further 25 µl was added to the first well of each row (ELISA lines). To the wells of the alternate, even-numbered rows (ELISA inhibition lines) was added 50 µl of phosphate-buffered saline with 0.05% Tween 20 containing 30 µg of antigen per ml; again, an additional 25 µl was added to the first wells of each of these rows. (The suspended antigen competes with the antigen bound to the plate for available antibody in the test serum). Testing then commenced by the addition of 25 µl of test serum, neat or prediluted, to the first wells of an ELISA line and its adjacent ELISA inhibition line. Twofold dilutions were made from the first wells along the length of the plate.

After 30 to 45 min on a plate shaker at 37°C the plate was washed, and 50 µl of a 1:800 dilution of peroxidase-conjugated anti-human (Dakopatts, Copenhagen, Denmark) or anti- guinea pig (Miles-Yeda, Rehovot, Israel) globulins, as appropriate, was added to all wells.
After a further 30 min of incubation at 37°C the plate was again washed, and 50 µl of substrate 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well. The plates were
held for a further 50 min at 37°C and read on a
MR 580 Microelisa Autoreader (Dynatech) at 405 nm. The highest of two or more consecutive dilutions showing a difference of
>20% between the ELISA and the ELISA inhibition lines was taken as the titration endpoint (32).

Ouchterlony gel diffusion tests for antibody. The methods of preparation of standard antigen and standard antiserum and of titration of antibody have been described in detail elsewhere (27). The current standard antiserum N17 requires a final dilution of 1:4 of the standard antigen. Thus 0.2-ml volumes of 1:2 standard antigen were mixed with 0.2-ml volumes of serial twofold dilutions of test serum before they were added to the antigen wells in the Ouchterlony plate.

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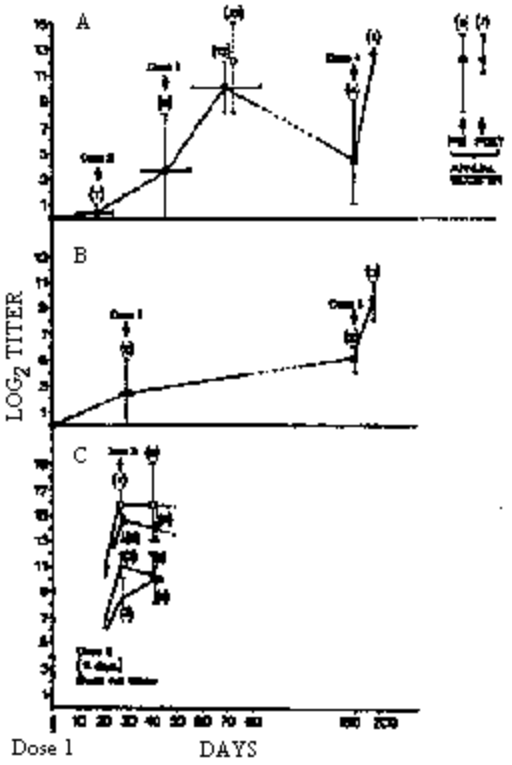

FIG. 1. The development of antibodies, as detected by ELISA, to protective antigen in vaccinated humans and guinea pigs. (A) Human vaccinees inoculated by the recommended schedule. (B) Human vaccinees in whom dose 3 was administered after a 6-month delay. (C) Guinea pigs vaccinated with human-type (chemical) vaccine and bled prior to dose 3 and to challenge at 28 and 42 days,
respectively, after dose 1. Points show IgG response to British (★, ⊡) and U.S. (○, □) vaccines and IgM response to British (π) and U.S. (ρ) vaccines. Each point is the mean of the titers of the number of individuals or animals shown in the parentheses against that point. The range of the titers within the group is indicated by the vertical bar through the point.

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The highest dilution of test antiserum that prevented formation of a visible precipitin line was taken as the endpoint.

IMHA. A modified version of the original method of IMHA (5) was used. Tanning of sheep erythrocytes was accomplished by adding 20 ml of 0.05 mg of tannic acid per ml to 20 ml of a 2.5% suspension of the erythrocytes and incubating in a water bath at 37°C for 10 min. Sensitization was done by suspension after three washes of half of the erythrocytes in 1 ml of buffered saline to which was added 75 μl of a 3.3 mg/ml of PA. The other,, unsensitized, control erythrocytes were treated in parallel with 1.075 ml of buffered saline. After the final suspension was held for 15 min at room temperature and overnight in a refrigerator, it was then washed twice in 30 ml of buffered saline containing 1% normal rabbit serum.

The sera under test were heat inactivated at 56°C for 30 min and adsorbed by the addition of one drop of packed washed sheep erythrocytes, followed by incubation at room temperature for 15 min. After centrifugation, duplicate 25-μl
samples were doubly diluted in adjacent rows in 96-well round-bottom plastic plates containing 25 μl of phosphate-buffered saline containing 1% normal rabbit serum per well
as diluent, and 25-μl volumes of sensitized and unsensitized erythrocytes were dispensed into the respective test and control rows. Hemagglutination titers were then read after overnight incubation at room temperature.

Rocket electrophoresis. Antisera to purified PA and LF were developed in rabbits to ELISA titers of 1:2.6 x 10^5. The proportions found suitable for rocket electrophoresis were 250 μl of the anti-PA and 200 μl of the anti-LF
antiserum dissolved in 12.5 ml of 1% agarose. Volumes (5 μl) of samples under test and purified PA or LF standards were inserted into the wells, and a potential difference of 7.5 V/cm was applied for 3 h.

Challenge. On completion of the appropriate vaccination schedule, the immunized guinea pigs were divided into groups for intramuscular challenge with 500 to 1,000 spores of each of four strains of *B. anthracis*, designated Vollum (guinea pig intramuscular
[i.m.] 50% lethal dose [LD_{50}], <10 spores; mouse intraperitoneal [i.p.] LD_{50}, 48.67 spores [95% confidence limits, 19.81 to 91.47]), Ames (guinea pig i.m. LD_{50}, <10 spores; mouse i.p. LD_{50}, 10.8 spores [95% confidence limits, 0.8 to22.0]), New Hampshire (guinea pig i.m. LD_{50}, <10; mouse i.p. LD_{50}, 50.7 spores [95% confidence limits, 30.0 to 87.0]) and penicillin resistant (guinea pig i.m. LD_{50}, <10 spores; mouse LD_{50}, not known). The LD_{50} readings were the data of M.G. Broster.

The animals vaccinated with British and U.S. human and Sterne live spore vaccines were divided into challenge groups of 11 or 12 animals each for each *B. anthracis* strain. Because a third of the animals vaccinated with the STI live spore vaccine succumbed to the vaccine itself, challenge groups of STI-vaccinated guinea pigs were restricted to six per *B. anthracis* strain. Groups of 12 unvaccinated control animals were also inoculated with each challenge strain. Survivors in any of the groups were held for 1 week before termination of the experiment.

Statistical analysis. Mean $\log_2$ antibody titers were compared by using Student's *t* test for two small samples. The survival rates of vaccinated guinea pigs following challenge were compared by using a 2 x 2 chi-squared test with Yates correction. Correlation coefficients were calculated to compare $\log_2$ titers of IMHA or Ouchterlony results with ELISA results for the same specimens and were tested for significant departures from zero by the exact *t* test.

RESULTS

Development of antibodies to PA and LF. Anti-PA immunoglobulin G (IgG) antibody titers determined by ELISA in the previously unvaccinated individuals receiving British-type vaccine followed a response curve that was entirely normal for a protein antigen (Fig. 1A and B). Evidence of antibody was apparent in the sera of some of the vaccinees at 2 to 5 weeks after dose 1, although a titer could only be assigned to one. In individuals vaccinated essentially in accordance with the recommended schedule (Fig. 1A), dose 2 resulted in antibody levels which ranged from negative to a titer of 1:256 at the time of administration of dose 3. Low but measurable titers were found (Fig. 1B) in two of three persons whose dose 3 was delayed until 6 months after dose 2 (the sera of the third individual was unavailable). Whether delayed or not, dose 3 resulted in titers in all individuals that ranged from 1:256 to 1:4,096. By the time the 6-month booster (dose 4) was due, titers had dropped appreciably but were again raised by this booster to 1:4,096 or 1:8,192 in all the sera tested. The titers in the two individuals given a short course of vaccination, consisting of half of the recommended dose at 10-day intervals, rose to levels comparable with those in the conventionally vaccinated persons.

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TABLE 1. ELISA-detected antibodies (IgG) to PA and LF after the most recent annual booster (British vaccine) in six unrelated persons

Time after booster (mo)	Titer of*:	
	Anti-PA	Anti-LF
3	8,192	4,096
6	4,096	4,096
9	2,048	1,024
12	8,192	16,400
18	8,192	1,024
60	4,096	16,400

* Titer expressed as the reciprocal of the dilution.

The pre- and postannual booster plots in Fig. 1A were derived from the sera collected in a single session for reasons not related to anthrax vaccination from 31 fully vaccinated persons. Of these persons, seven had received an annual booster <6 weeks before that date (postbooster), and five were due for another (prebooster). The mean titers of these groups were both 1:4,096. Only two of these persons, who had received their last annual booster 0.5 to 60 months before, had titers of <1:1,024, and there was no general decline of titer with time (Table 1). Titers after dose 4, therefore, appeared to settle at a level which remained steady for an indefinite period. Sera available from one individual who had received annual boosters for many years showed prebooster titers of 1:1,024 or 1:2,048 and postbooster titers of 1:4,096 or 1:8,192.

In British vaccinees, the anti-LF response paralleled the anti-PA response (Fig. 2 and Table 1). None of the sera obtained from the U.S. vaccinees after dose 3, however, had detectable anti-LF antibody.

IgM antibodies to both PA and LF were not detected in any of the human sera represented in Fig. 1A and B and Fig. 2. This may simply reflect that the doses in the human vaccinees were too small to elicit detectable levels of IgM antibody. Such antibodies were detected in the guinea pigs to which the human vaccines were given (Fig. 1C), but in terms of body weight, these animals received 100- to 200-fold greater doses than the human vaccinees and they also developed appreciably higher IgG titers than the humans. The mean IgG and IgM anti-PA titers in animals vaccinated with the U.S. vaccine were 1 to 2 titration units higher than those vaccinated with the British vaccine. As in the human vaccinees, anti-LF antibodies were as evident as anti-PA antibodies in guinea pigs that had received the British vaccine but were far less evident (P <0.002), if detectable at all, in the animals given the U.S. vaccine (Fig. 3).

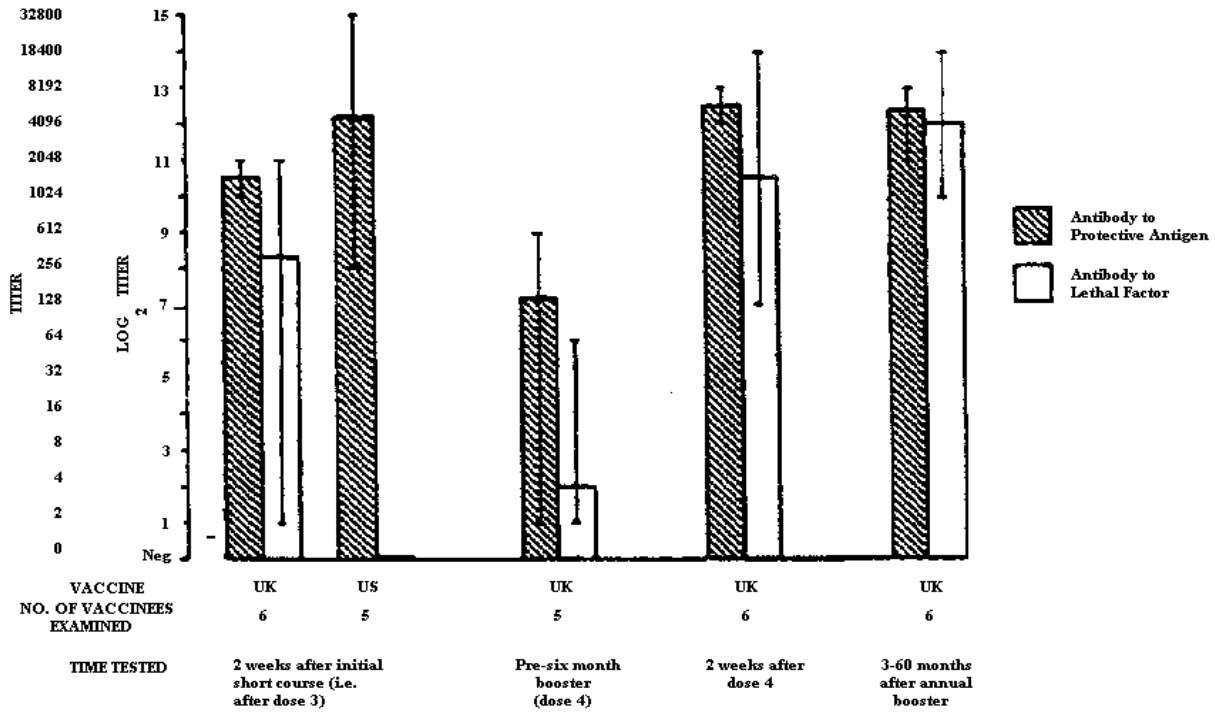

Fig. 2. ELISA-detected antibodies (IgG) to protective antigen and lethal factor in vaccinated humans. The height of the box represents the mean titer of the group, and the range is indicated by the vertical bars. IgM antibodies were looked for but not detected.

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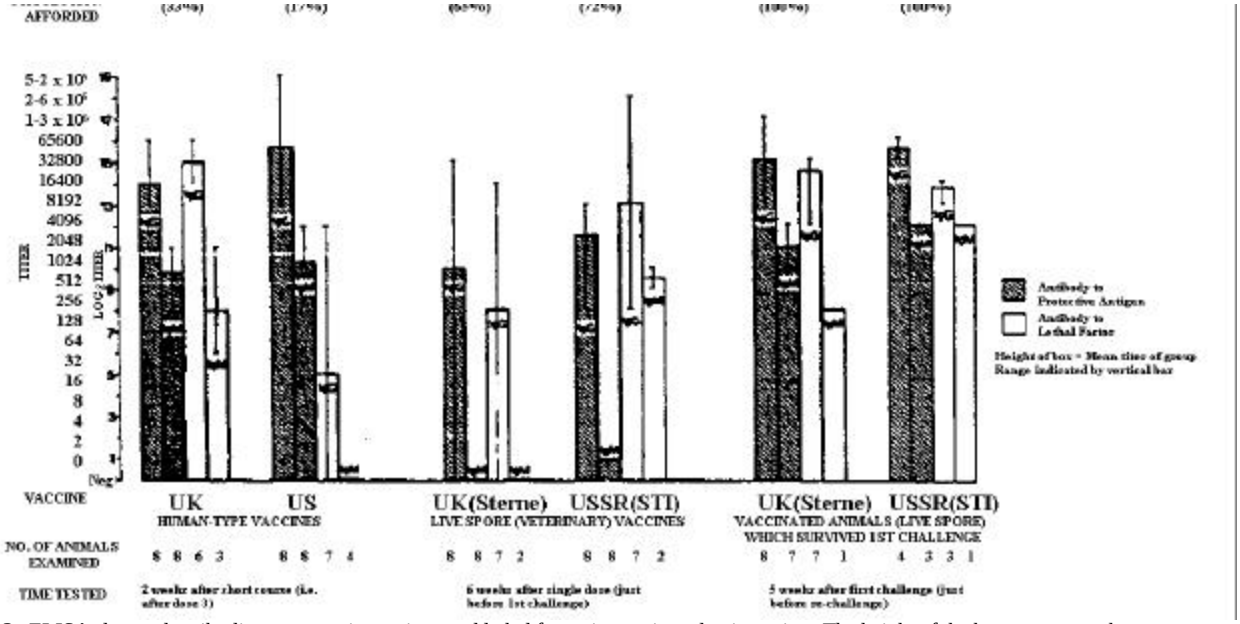

FIG. 3. ELISA-detected antibodies to protective antigen and lethal factor in vaccinated guinea pigs. The height of the box represents the mean titer of the group with the range indicated by the vertical bars. Values at the top of the figure are the number of survivors per number challenged (%) and indicates the protection afforded against challenge with 500 to 1,000 spores of Ames, New Hampshire, and penicillin-resistant strains. All vaccines gave solid protection against similar challenge with the Vollum strain. All of 48 unvaccinated control guinea pigs (12 per strain) succumbed to challenge.

Single doses of the live spore vaccines in the guinea pigs resulted in generally lower anti-PA IgG and IgM titers than had been induced by the short course of human-type vaccines *(P* <0.0005; Fig. 3). Anti-LF IgG antibody levels were lower in the live spore-vaccinated animals than in the group that had received the British human-type vaccine but were higher than those that had been given U.S. human-type vaccine. The Russian STI strain elicited higher mean titers of both antibody classes to both toxin components than the Sterne strain (not statistically significant for PA; *P* <0.02 for LF). However, the STI strain was relatively virulent and killed almost a third of the guinea pigs to which it had been given as a vaccine.

Four animals from each of the Sterne and STI groups were tested 21 days after inoculation and had mean anti-PA IgG titers that were <1 titration unit lower than the mean values shown in Fig. 3 (IgM antibodies were not detected). It is likely, therefore, that the 6-week IgG readings represent the maximum titers reached following the single-dose live spore injection. Challenge with virulent anthrax resulted, after a further 5 weeks (Fig. 3), in high levels of anti-PA and anti-LF (both IgG and IgM) antibodies in both Sterne and STI strain-vaccinated survivors.

Antibody levels and protection. The groups of guinea pigs that received human-type vaccine proved to be significantly less protected *(P* <0.001) than the groups that received the live spore vaccines, despite the fact that they had generally higher levels of anti-PA IgG and IgM antibodies and, in the group given British human-type vaccine, as high or higher anti-LF titers as well (Fig. 3). Antibody levels in the rechallenged animals, which proved to be solidly (100%) protected, however, were consistently and well elevated. Also of consequence was the finding that solid protection was provided by all the vaccines on initial challenge against the Vollum strain of *B. anthracis*.

TABLE 2. ELISA versus IMHA for assay of antibodies (all immunoglobulins) to PA in vaccinees

Developing antibodies in new vaccinees						Fully vaccinated individuals[a]			
British vaccine[b]			U.S. vaccine[c]						
Serum sample	Titer by IMHA[d]	Titer by ELISA[d]	Serum sample	Titer by IMHA	Titer by ELISA	Serum sample	No. of months since last booster	Titer by IMHA[d]	Titer by ELISA[d]
PT1	Neg	Neg	US7	64	4,096	JC	0.5	32	2,048
PT2	Neg	Neg	US9	64	2,048	CW	1.5	256	16,400
PT3	32	1,024	US13	16	4,096	DJ	1.5	128	4,096
PT4	64	1,024	US15	32	2,048	BX	3	128	8,192
PT5	16	512	US19	32	8,192	BZ	5	256	8,192
PT6	2,048	8,192	US20	8	4,096	AE	5	64	4,096
CT1	Neg	Neg	US21	2	2,048	DT	8	256	8,192
CT2	Neg	Neg	US24	4	2,048	CZ	9	64	2,048
CT3	±	128				DB	12	512	8,192
CT4	4,096	4,096				CH	41	128	4,096
						AD	58	512	4,096

[a] All had undergone the full vaccine course, including at least one annual booster (British vaccine).
[b] PT1 to PT6 and CT1 to CT4 are sera from two previously unvaccinated individuals taken at appropriate intervals during their course of vaccination. All other sera are from unrelated vaccinees.
[c] End of short course.
[d] Titer expressed as the reciprocal of the dilution. Neg. Negative titer.

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ELISA and Ouchterlony gel diffusion test. Detectable antibody (titers: neat, 1:2 and 1:4 or, rarely, 1:8) in the Ouchterlony test generally corresponded with a substantial ELISA titer (>1:2,048) in any one serum specimen, and boosted Ouchterlony titers were reflected in correspondingly raised ELISA titers (correlation coefficient, 0.45; $P < 0.05$). In a high proportion of specimens (57%), however, antibody levels were below those detectable by the Ouchterlony test.

ELISA and IMHA. Comparative IMHA and ELISA titers in the sera of vaccinees and patients with clinical anthrax in Zimbabwe (Tables 2 and 3) showed that there was a strong positive correlation between the two tests as applied to sera from 29 vaccinated humans (correlation coefficient, 0.63; $P < 0.0005$), 21 vaccinated guinea pigs (correlation coefficient, 0.57; $P < 0.005$), and 31 human anthrax patients (correlation coefficient, 0.59; $P < 0.005$). The overall correlation coefficient was 0.67.

PA and LF levels in human vaccines. Rocket electrophoresis of cell-free culture filtrates of the Sterne strain grown under the conditions specified for the British chemical vaccine and of the complete alum-precipitated vaccine (treated 1:1 with 0.1 M citric acid to dissolve the alum [26]) indicated that British vaccine PA and LF levels were of the order of 50 and 15 μg/ml, respectively; evidently, the alum precipitation process efficiently captures the antigens with minimal losses. Attempts to separate the antigen from aluminum hydroxide in the U.S. vaccine were unsuccessful, and PA and LF levels in this vaccine could not be determined.

Antibodies in sera of patients with anthrax. ELISA evidence of anti-PA antibody was found in 27 (35%) of 77 sera from the individuals clinically diagnosed in Zimbabwe as having anthrax. The range of titers found is apparent in Tables 3 and 4, which list the results for those sera used in the additional tests shown. Of the 15 sera also tested for anti-LF antibody, the 5 that were negative for anti-PA were also negative for anti-LF, and 5 others were also negative. The remaining five sera exhibited clear anti-LF titers (Table 4).

DISCUSSION

The antibody responses to PA and LF in humans and guinea pigs were normal for protein antigens. As responses to anthrax vaccines have not been fully monitored before, little was known hitherto about the development of antibodies to PA, long thought to have a primary role in protective immunity, when administered preformed in chemical vaccines or when synthesized in situ following administration of live spore vaccines. Furthermore, little was known about the LP content of chemical vaccines, its in vivo production following administration of live spore vaccines, and its antigenic activity in vaccinated humans and animals.

TABLE 3. IMHA and ELISA anti-PA antibody titers (all immunoglobulins) in the sera of 31 persons diagnosed clinically as having anthrax.

Patient	Titer by[a]	
	IMHA	ELISA
11 cases	Neg	Neg
10 cases	Neg	32-1,024
4 cases	± 4	Neg-256
017	16	256
05	16	2,048
N21	32	1,024
032	64	2,048
N68	1,024	1,024
N32	>16,400	>16,400

[a] Titers expressed as the reciprocal of the dilution. Neg. Negative

TABLE 4. ELISA anti-PA and anti-LF antibody titers (all immunoglobulins) in the sera of 15 persons diagnosed clinically as having anthrax.

Patient	Titers of[a]:	
	Anti-PA	Anti-LF
5 cases	Neg	Neg
N7	±	1,024
N10	64	Neg
N69	64	Neg
N118	128	1,024
N66	256	8,192
N9	512	Neg
N68	1,024	Neg
N82	1,024	Neg
N21	1,024	128
N32	>16,400	8,192

[a] Titers expressed as the reciprocal of the dilution. Neg. Negative.

Inherent in the production methods has been the likelihood that the U.S. vaccine, which is produced from cultures grown anaerobically in a defined medium (19, 33), has a higher and relatively more pure PA content than the British vaccine, which is derived from cultures grown aerobically in an undefined medium (27). The results presented here appear to support this contention. The U.S. vaccine produced higher anti-PA titers in both humans and guinea pigs ($P < 0.1$) than the British vaccine (Fig. 1C, 2, and 3). Also, while humans and guinea pigs receiving the British human vaccine developed readily detectable anti-LF antibodies (Fig. 2 and 3), such antibodies were not detected in the U.S. vaccinees (Fig. 2) and were only detected at significantly lower titers ($P < 0.002$) in five of seven of the guinea pigs that received the U.S. vaccine (Fig. 3). The guinea pigs received 100- to 200-fold greater doses than the humans, and this probably accounts for the generally higher IgG responses in the guinea pigs to the human-type vaccines, as compared with that in the humans, and for the detection of IgM antibodies only in the guinea pigs. Rocket electrophoresis confirmed the LF content of the British vaccine, but the U.S. vaccine could not be similarly analyzed.

The antibody responses in the two individuals given one-half the doses of the British vaccine over a shorter time period were indistinguishable from responses in persons given the recommended dose by the currently prescribed schedule. This indicates that the doses and time intervals in the initial short course are not critical and could probably be reduced without deleterious effects. On the other hand, the relatively poor protection apparently afforded by the human-type vaccine in guinea pigs raises the question of the relevance of the measured immune response in humans.

The annual booster had an unremarkable effect on the persisting titers of human vaccinees; pre- and postannual booster titers differed by only 1 to 4 titration units. However, these boosters may stimulate other undefined protective mechanisms, and their continuation is advised until more information is available.

Compared with the human chemical vaccines, the live spore vaccines resulted in greater protection ($P < 0.001$) while associated at the time of challenge with lower anti-PA and, in the case of the UK vaccines, lower anti-LF titers ($P < 0.0005$; Fig. 3). On the other hand, solid protection in the animals rechallenged 5 weeks after they survived the first challenge was associated with the highest IgG and IgM titers that were obtained (Fig. 3). It will be necessary to hyperimmunize animals with purified toxin components to determine whether complete protection can be provided solely by antibody to PA or LF or both, as long as they are present in

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sufficient quantities. In fact, one guinea pig, which had been vaccinated with British live spore vaccine and from which blood was obtained shortly after death following challenge, was found to have succumbed despite the fact that it had very high anti-PA (IgG, 1:1.2 x 10^5; IgM, 1:1,024) and anti-LF (IgG, 1:65, 600; IgM, 1:512) titers. Although confirmation of this finding is needed in other animals, the inference is that effective protection will prove to be a function of more than just substantial antibody titers to PA and LF.

When purified edema factor, the third anthrax toxin component and a calmodulin-dependent adenylate cyclase (12), is available in sufficient quantities, its role will also be investigated.

If, as has been proposed (15, 16), the original Pasteur vaccine strains had been cured of plasmids coding for production of the toxin components by heat attenuation, then their efficacy was not dependent on antibodies to these components. The theory that these strains were cured of their toxin-related plasmids, however, has been challenged now (29).

The capsule is the other known major virulence factor of *B. anthracis*, but the effective immunity produced by the noncapsulated live spore vaccines indicates that this antigen does not play a significant role in protective immunity.

The greater effectiveness of the live vaccines compared with that of the human vaccines may be the result of the involvement of antigens other than PA, LF, and purified edema factor in protective immunity. Alternatively, the dynamic relationship between the toxin components may be all important (8, 13); perhaps, for maximal effect, the toxin components must be presented to the immune system at a certain rate, concentration, and order. It is possible that the live vaccines come closer than the chemical vaccines to achieving this. The recent observation (17) that purified edema factor in combination with PA inhibits the phagocytic activity of neutrophils highlights the need to examine protective activity of vaccines in the light of cellular as well as humoral immunity.

The ELISA system also detected anti-PA antibodies in the sera of a number of individuals who had been diagnosed as having clinical anthrax (Table 3). The majority (65%) of sera from such cases, however, were negative. This may represent sera collected at inappropriate times, early and rapid elimination of antigen by antibiotic treatment, incorrect diagnosis, or responses to some cutaneous lesions that were too weak to permit detection by the present techniques. Clinical details available on a proportion of these sera and the associated serological analysis will be published elsewhere.

Anti-LF antibody was detected in 5 of 10 patients whose sera showed evidence of anti-PA antibody (Table 4). Unlike the humans and guinea pigs that received British human-type vaccine and the guinea pigs that were given live spore vaccines, the anti-LF titers in the sera from humans did not appear to parallel the anti-PA titers especially closely, although all five sera that were negative for anti-PA were also negative for anti-LF. The volumes of sera available were very limited, however; and further detailed analysis of this apparent lack of parallel awaits the availability of more sera.

ELISA proved to have numerous advantages over existing methods for the measurement of antibody to anthrax antigens. It is much more sensitive and rapid than the Ouchterlony test which, for all practical purposes, can now be discarded. IMHA is probably not a great deal less sensitive than ELISA, but in agreement with a previous report (11), its reproducibility was found to be very poor, the sheep erythrocytes varied greatly from one blood collection to the next, and erythrocyte-antigen preparations were unstable. Furthermore, titration endpoints required subjective judgments.

The main drawback of the competitive inhibition ELISA is the requirement for fairly large quantities of purified antigen (±45 μg of PA or LF per single test), but its advantages are simple machine-read endpoints (based on a 20% difference between ELISA and ELISA inhibition lines representing >3 standard deviations from the means and a chance of false positives of <1% [32]) with excellent reproducibility and high confidence in the specificity of the test. Stability of the antigen-coated plates and the speed and simplicity of performance of the test are additional assets.

On the basis of already attainable production levels (S. H. Leppla, personal communication) of 3.3 mg of PA per ml (±70 tests per ml) and 1.56 mg of LF per ml (±35 tests per ml), the ELISA inhibition test is entirely adequate for routine test loads that might be anticipated in reference or clinical laboratories. In fact, for purposes of monitoring vaccine performance or confirmation of infection on a routine basis, an anti-PA assay would appear to be sufficient; there is no obvious need to include anti-LF assays.

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