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Comparison of Enzyme-Linked Immunosorbent and Indirect Hemagglutination Assays for Determining Anthrax Antibodies

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An enzyme-linked immunosorbent assay has been established to measure anthrax antibody titers. The protective antigen component of anthrax toxin was used as the capture antigen. Two types of conjugates (protein A-horseradish peroxidase and anti-human immunoglobulin G plus immunoglobulin A plus immunoglobulin M-horseradish peroxidase) were tested. Results from enzyme-linked immunosorbent assay testing were compared with those from indirect hemagglutination titers on serum from vaccinees. The overall trend of enzyme-linked immunosorbent assay and indirect hemagglutination titers was significant. The enzyme-linked immunosorbent assay offered speed, precision, and reduced cost per test.

The development of a safe anthrax vaccine by Puziss et al. (7, 8) and Wright and his co-workers (12) led to a dramatic decline in the number of illnesses reported in humans each year. A subsequent field trial, conducted by Brachman et al. (1), established epidemiological evidence that this vaccine was efficacious in preventing cutaneous anthrax in an industrial setting.

In 1971, Buchanan et al. (2) published a new method for detecting anthrax antibodies in sera. The indirect hemagglutination assay (IHA) that they described was a great improvement over the modified agar-gel precipitation assay originally used in the 1960s (9, 10). The IHA was more sensitive and provided a significant increase in the rapidity of testing. Certain drawbacks in this assay, however, include lack of reproducibility, long time required for preparation, lack of availability of sheep erythrocytes, and short usable life of the sensitized erythrocyte-antigen preparations.

In this paper, a report is presented on adaptation of an enzyme-linked immunosorbent assay (ELISA) for detection of anthrax antibodies, using the protective antigen (PA) component of anthrax toxin. Vaccinees were monitored for antibody production after receiving an initial series of immunizations and after a yearly booster. Results from the ELISA were compared with IHA titers on identical serum samples.

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MATERIALS AND METHODS

Antigen. The anthrax PA, one component of the three-part anthrax toxin, was prepared by the Michigan Department of Public Health, as previously described (6). Briefly, the avirulent strain V770-NP1-R of *Bacillus anthracis* was grown overnight at 37°C in a chemically defined medium (7) under microaerophilic conditions. Bacteria were removed by filtration through glass candles, and the culture supernatant (adjusted to pH 8.0) was passed over a column containing Dowex 1-XI resin (adjusted to pH 8.0). The antigen bound to the resin was subsequently eluted with 1 M NaCl. Fractions from the column were tested specifically for the PA by agar-gel diffusion, using crude antiserum (10). Positive fractions were pooled and frozen in 5-ml samples. The antigen was stored at −70°C, thawed, and dialyzed at 4°C against phosphate-buffered saline (PBS; 10 mM, pH 7.1) before use. The purity of the antigen was analyzed by polyacrylamide gel electrophoresis.

Gel electrophoresis. An acrylamide-bisacrylamide mixture (30:0.8) (Bio-Rad Laboratories, Richmond, Calif.) was polymerized to form a running gel of 10% acrylamide, with 0.1% sodium dodecyl sulfate added. Electrophoresis buffer was made according to Laemmli (4). Samples were denatured by heating to 100°C for 10 min in the presence of 1% sodium dodecyl sulfate. Electrophoresis in slabs (thickness, 1.5 mm) was continued until the tracking dye reached the bottom of the gel (ca. 5 h). Proteins were stained with a solution of 0.25% Coomassie brilliant blue R-250, made fresh in the destain solution (acetic acid, methanol, water; 1:5:5). Molecular weight standards were obtained from Bio-Rad Laboratories.

Vaccine. Anthrax vaccine, adsorbed, was produced by the Michigan Department of Public Health. The product contained extracellular materials elaborated during the growth of an avirulent, nonencapsulated strain of *B. anthracis*. The vaccine was adsorbed with aluminum hydroxide, benzethonium chloride as a preservative, and formaldehyde as a stabilizer.

More than 100 employees at this laboratory were immunized with 0.5-ml doses of the vaccine, given at the intervals recommended by the distributor (Centers for Disease Control, Atlanta, Ga.). Serum samples were obtained from all employees after three biweekly injections of the vaccine; further serum samples were received on a voluntary basis.

Antiserum. Partially purified antigen (Dowex column eluate [described above]) was used to immunize two New Zealand rabbits. The first two injections contained Freund complete adjuvant, and subsequent injections were made with the soluble antigen (50 μg per immunization). Serum was obtained 2 weeks after a series of six injections, given at 2- to 3-week intervals.

Crude antiserum was prepared by injecting two burros with live spores of the avirulent Sterne strain of *B. anthracis*. Immunizing doses were gradually increased from 10^6 to 10^{10} spores and were given in the cervical area. Serum was obtained 2 weeks after a series of 6 monthly injections.

Commercially available normal human serum (Fisher Scientific Co., Orangeburg, N.Y.) was used undiluted as a negative control serum for the human serum titers in the ELISA and IHA. In addition, serum from each subject (two

rabbits, two burros, and 154 employees) was tested in both assays before any immunization. All preimmune samples were negative for antibody in these tests.

Conjugates. Protein A-horseradish peroxidase was obtained from Sigma Chemical Co., St. Louis, Mo. It was used at a 1:500 dilution in the ELISA. Anti-human immunoglobulin G (IgG) plus IgM plus IgA coupled to horseradish peroxidase, obtained from Miles Laboratories, Inc., Elkhart, Ind., was used at a 1:10,000 dilution.

Substrate. ABTS substrate [2,2'-azino-di (3-ethyl-benzthiazoline sulfonic acid) diammonium salt] was obtained from Sigma Chemical Co. and was made fresh before use at a concentration of 1 mg/ml in 0.1 M sodium citrate (pH 4.0). Hydrogen peroxide (freshly diluted to a final concentration of 0.003%) was added immediately before use. Alternatively, *O*-phenylenediamine, also obtained from Sigma Chemical Co., was made up to 0.4 mg/ml in 0.1 M phosphate-citrate buffer (pH 5.0). Again, hydrogen peroxide was added immediately before use. The color reactions were stopped by the addition of 37 mM sodium thiocyanate or 4 N sulfuric acid for the ABTS substrate or *O*-phenylenediamine, respectively. Color intensity was measured in a Titertek Multiscan (Flow Laboratories, Inc., McLean, Va.) ELISA reader at 414 and 492 nm, respectively, for the ABTS and OPD.

ELISA procedure. Microtiter plates (96 well, flat bottom; Becton Dickinson and Co., Paramus, N.J.) were coated with 100 μl of the PA (diluted to 5 μg/ml in 0.05 M sodium borate buffer [pH 8.9]). Plates were incubated at 37°C for 90 min. Additional binding sites on the wells were blocked by adding 200 μl of bovine serum albumin solution (1 mg/ml) in borate buffer. The plates were incubated for 90 min at room temperature and then washed four times with PBS containing the detergent Nonidet P-40 (Sigma Chemical Co.) made up to a concentration of 0.05%. Test sera and control sera were diluted (fourfold dilutions) in the wells, using 0.05% Nonidet P-40–PBS as a diluent. After sera were added, the plates were incubated for 2 h at room temperature and then washed four times with Nonidet P-40–PBS. Next, 100 μl of either protein A-horseradish peroxidase or anti-human IgG plus IgM plus IgA-peroxidase conjugate was added per well and incubated for 30 min at room temperature. After four washes with Nonidet P-40–PBS, 100 μl of substrate was added to each well.

IHA. Sera were screened for antibody by using the partially purified antigen coated on tanned sheep erythrocytes as described by Buchanan et al. (2). Sera were heat inactivated (56°C for 30 min) and absorbed with sheep erythrocytes before testing.

RESULTS

IHA titrations. Serum samples were received from vaccinated employees 2 weeks after they had received an initial series of three anthrax immunizations (i.e., 0, 2, and 4 weeks). Anthrax antibodies were first measured by the IHA (Fig. 1). If a titer of 1:8 or above is considered positive, 83% of the vaccinees seroconverted.

Serum samples were also obtained from some of these employees before a yearly booster immunization and again 2 weeks after the booster immunization. Results (Table 1) indicated that a high percentage of vaccinees had little or no measurable titer at the time of the booster immunization, but they did demonstrate an anamnestic response after receiving the vaccine.

Antigen analysis. Acrylamide gel electrophoresis of the PA preparation used in these studies indicated that the major protein band had a molecular weight of about 85,000, as estimated from the molecular weight markers (Fig. 2). Several other low-molecular-weight peptides were detected in much lower concentration. Identical results were obtained in two different buffer systems (data not shown). In addition, the PA gave a single band of precipitation when tested with crude antiserum. From these analyses, the antigen was estimated to be ≥95% pure.

ELISA. The ELISA was used to determine the titers of

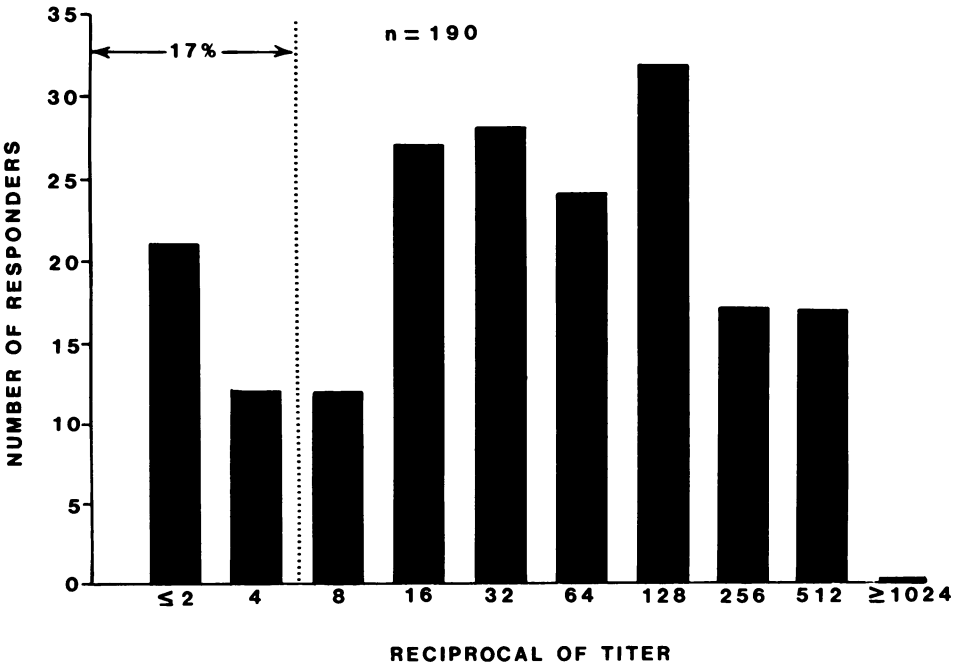

FIG. 1. Anthrax antibody titers measured by IHA on 190 vaccinees. Serum samples were obtained 2 weeks after the third immunization in the anthrax series. Titers of 1:8 or higher were considered positive. A total of 83% of vaccinees tested had seroconverted to the vaccine.

TABLE 1. IHA titers of vaccinees

Titer[a]	No.	%
Before immunization		
≤2	27	47.0
4	9	15.8
8	10	17.5
16	2	3.5
32	4	7.0
≥64	5	8.8
After immunization		
≤2	0	0
4	0	0
8	2	2.4
16	2	2.4
32	3	3.5
64	3	3.5
128	11	12.9
256	10	11.8
512	17	20.0
1,024	8	9.4
2,048	13	15.3
≥4,096	16	18.8

[a] Before immunization, immediately before annual immunization (n = 57); After immunization, 2 weeks after annual immunization (n = 85).

serum samples from more than 100 vaccinees at various stages of the immunization regime, including some samples before immunization as well as samples from personnel receiving seven or more immunizations. When the fourfold dilution scheme was used for the serum samples in the ELISA, 21 samples (undiluted) showed essentially the same optical density as that of the negative control serum; these were recorded as negative titers. An additional 23 samples (diluted 1:4) and 14 samples (diluted 1:16) produced an optical density within 1 standard deviation of that of the mean negative control serum. All other titers (recorded as 1:16 or greater) showed optical density readings of ≥2 standard deviations higher than that of the negative control. The highest dilution of test serum giving this reading was recorded as the titer. Figure 3 shows a comparison of 154 serum titrations in both the ELISA and the IHA. More than 75% of the samples tested matched in both tests, i.e., were positive in both or negative in both assays.

Each assay system (IHA and ELISA) gave essentially an equal number of responses above the thresholds: (i) IHA had 94/154 (61%) with a titer of >1:4, and (ii) ELISA had 96/154 (62%) with a titer of >1:16.

In addition, each procedure gave an equivalent percentage of false-positives, i.e., 18 samples were IHA positive and ELISA negative, and 20 samples were IHA negative and ELISA positive.

The overall linear trend of all IHA versus ELISA titers was significant (r, 0.47; P, <.001). However, there was no significant correlation when only the positive response region was considered. In other words, titers that were positive for both IHA and ELISA were not significantly correlated.

Reproducibility of the ELISA is depicted in Fig. 4. Samples of a known positive serum sample were retested on 5 different days, with multiple (two to three) assays per day. The mean ± standard error of the optical density readings for the various serum dilutions tested depicted a high degree of reproducibility (+6%) in the test system. Identical titers were obtained for a given sample when either the protein A-horseradish peroxidase or the anti-human IgG plus IgA plus IgM conjugate was used.

DISCUSSION

Use of the ELISA as described above will provide a more rapid and more simplified assay for measuring anthrax antibody in serum samples. Advantages of the ELISA (as reported for many other similar procedures) include sensitivity, no need for specialized equipment, small amounts of reagents used, and availability of enzyme-linked conjugates and substrates.

The ELISA reported here can be completed in ca. 7 h. The titers of ca. 100 serum samples can easily be determined by one technician during 1 work day. The reproducibility of the test is good, and the stability of the reagents makes the ELISA more attractive than the IHA. Both test systems used purified antigen, but the ELISA consumed only 50 μg of PA per microtiter plate (sufficient to determine the titers of 12 samples), whereas the IHA test used 50 μg of PA to coat enough sheep erythrocytes to determine the titers of 8 samples (a 50% increase in antigen consumption). The variability of the sheep erythrocyte source is eliminated by use of the ELISA. The conjugates used are available commercially, facilitating interlaboratory standardization. Neither test was affected by nonrelated antigens such as capsule

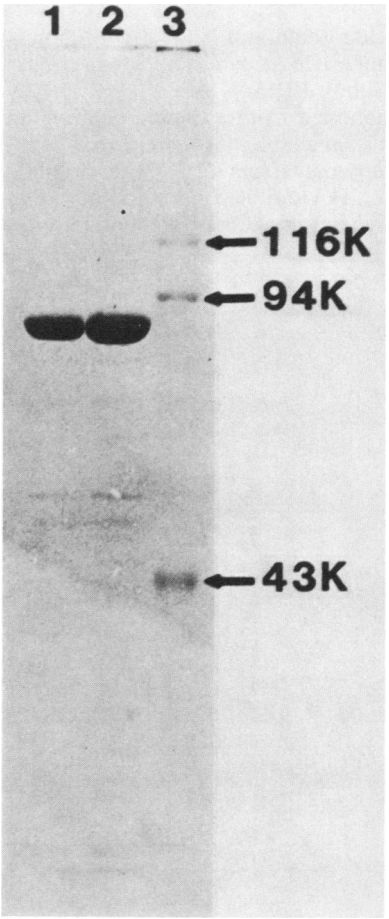

FIG. 2. Acrylamide gel (10%) run with sodium dodecyl sulfate. The anthrax PA was shown to be a peptide of about 85,000 daltons. Purity exceeded 95%. Lanes: 1, 10 μg of PA; 2, 20 μg of PA; 3, molecular weight markers.

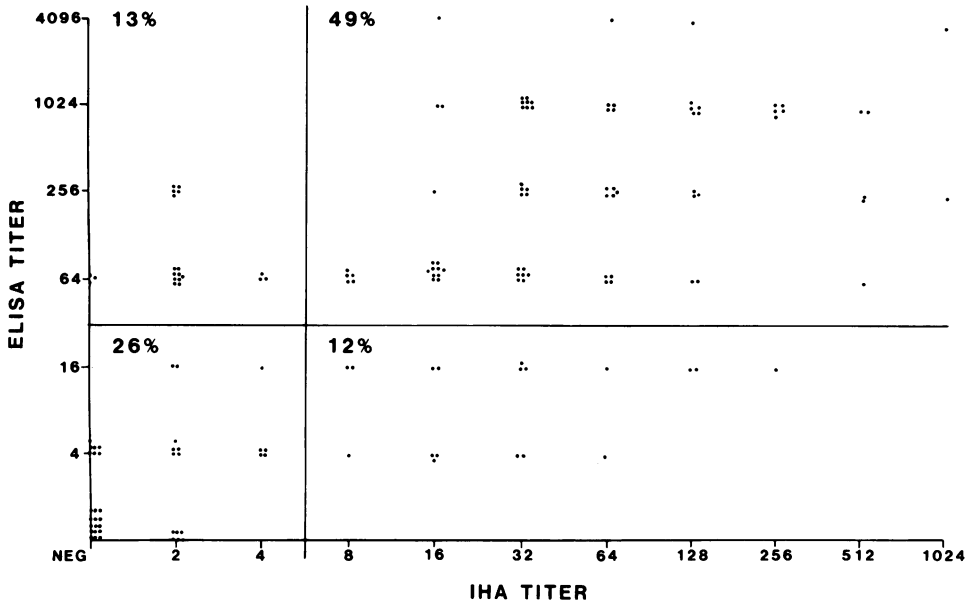

FIG. 3. Scattergram comparison of identical serum titers in the IHA (abscissa) and the ELISA (ordinate). ELISA titers of >1:16 were considered positive; 49% of samples tested were positive in both assays, and 26% were negative in both tests. ELISA data shown here are results with protein A-horseradish peroxidase and ABTS substrate.

polysaccharide (data not shown). Technicians trained to perform other ELISAs can easily adapt to the specifics of an anthrax antibody ELISA.

Results obtained from a comparison of this ELISA with the currently available IHA indicated a high correlation between the two systems (r, 0.47; P, <.001). The disadvantages of the IHA coupled with the advantages of ELISA make a significant impact on selection of a system for future testing. It has been reported previously that the anthrax vaccine evokes only low-level immunity (5, 9), and therefore, a more sensitive test system would be advantageous.

It is interesting to note that both the IHA and ELISA gave false-positive results when identical samples were assayed in both test systems. This may suggest that neither system is optimal for detecting low levels of antibody. However, the absence of any sign of clinical illness in our experience with

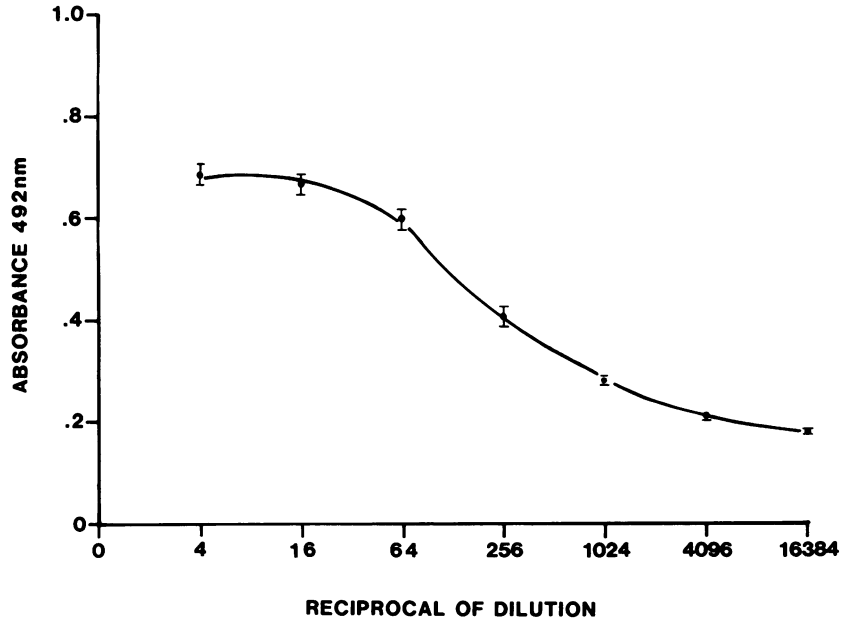

FIG. 4. Repeated titrations of a known positive serum sample in the ELISAs (using O-phenylenediamine substrate). The optical density (492 nm) of each well ± standard errors (n = 18) was plotted against the serum dilution. The negative control serum gave an average optical density of 0.200.

more than 100 vaccinated employees observed over a period of 4 years seems highly indicative of a satisfactory level of immunity in those individuals.

Currently, anthrax vaccine is administered routinely to workers who process wool, hair, or skins imported from areas where anthrax may be endemic, as well as to some laboratory workers considered to be at risk. The vaccination of these persons is the primary means of preventing anthrax in the United States. According to the World Health Organization (11), there were more than 1,500 cases of anthrax in 1977, the last year for which statistics have been published. There have been only a few widely scattered cases of human anthrax in the United States, recently attributed to products imported from Haiti (3). The continued use of anthrax vaccine is certainly advisable to avoid any chance of a large-scale epidemic. The ELISA can be used to monitor antibody titers in vaccinees and possibly as a basis for evaluating any new vaccines or immunization schedules that may be developed. The ELISA can also be adapted to screen for antibodies that may be produced against other antigens (edema factor, lethal factor, or other extracellular proteins), either with the currently available vaccine or with new products that may be developed.

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