Comparison of Passive Hemagglutination with Turkey Erythrocyte Assay, Enzyme-Linked Immunosorbent Assay, and Counterimmunoelectrophoresis Assay for Serological Evaluation of Tetanus Immunity

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Antibody titers to tetanus toxin in human sera were assayed by passive hemagglutination with turkey erythrocytes, enzyme-linked immunosorbent assay, and counterimmunoelectrophoresis. The first two of these tests were shown to be the most sensitive for antibody detection, having the same range of sensitivity and reproducibility. The antibody levels determined by these assays were up to 400-fold higher than those determined by counterimmunoelectrophoresis. The turkey erythrocyte hemagglutination assay requires only 40 min, whereas the immunosorbent assay method requires 24 h. These results suggest that the hemagglutination assay is the more appropriate method for rapid and sensitive determination of tetanus antibody levels.

Immunity against tetanus infection can be determined by the neutralization test (NT); however, this *in vivo* antibody titration method is very difficult to apply for screening purposes. Therefore, the passive hemagglutination (HA) test with tetanus toxoid adsorbed on sheep erythrocytes was developed by our laboratory and by others (1, 2, 7).

Furthermore, counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA) were proposed as assays for the evaluation of toxin antibody levels (19, 20). Recently, because of the limited sensitivity of sheep erythrocyte HA, we modified the passive HA assay to use turkey erythrocytes (TRBC) instead of sheep erythrocytes (11). This method is much more sensitive than with sheep erythrocytes and affords rapid determination of tetanus antibody levels.

The aim of the present study was to compare the TRBC-HA assay with CIE and ELISA assays to establish which is the most suitable for the rapid determination of tetanus antibody levels. Our data show that TRBC-HA has a higher sensitivity than the CIE assay and is more rapid than the ELISA.

MATERIALS AND METHODS

Passive HA test. The passive HA test of Mai and Rosin (10) with the modification of Marconi et al. (11) was used. Briefly, the test was carried out as follows. (i) An 8% suspension of TRBC, after being washed three times with a phosphate buffer (S-I buffer) con-

taining (grams per liter) NaCl, 4.8; Na₂HPO₄, 5.6; and KH₂PO₄, 1.45 (pH 7.4); was incubated with an equal volume of 3% Formalin in S-I buffer for 18 to 20 h at 37°C. (ii) After formalinization, excess Formalin was removed by repeated washing in twice-distilled water. Formalinized TRBC were incubated for 30 min at 56°C with a solution of tannic acid diluted 1:40,000 in phosphate buffer (S-II buffer) containing (in grams per liter) NaCl, 4.5; Na₂HPO₄, 3.3; and KH₂PO₄, 6.91 (pH 6.4). (iii) After the tannic acid treatment, TRBC were extensively washed in S-II solution. Purified tetanus toxoid solution (32 limes flocculation [Lf]/ml in S-II solution) was mixed with packed TRBC to obtain a 4% TRBC final solution and incubated for 2 h at 37°C. (iv) After sensitization was complete, the suspension of TRBC was washed with 0.5% NaCl solution and freeze-dried. (v) The test was performed on microhemagglutination plates (Cooke Microtiter) with 50 µl of TRBC (1.5×10^6 cells per ml) added to 50 µl of S-II-diluted serum. The covered plates were incubated at room temperature, and results were recorded after 40 min.

The international standard for tetanus antitoxin (Statens Serum Institut, Copenhagen, Denmark) was used as a reference. The unknown sera were measured against the reference serum. The antitoxin content was expressed in HA units (HU); 1 HU corresponds to 1 IU of the reference serum in the HA assay.

NT titers in mice. Titration of tetanus antitoxin was carried out at a toxin level of 1 L+/400/50, i.e., the amount of toxin which, if mixed with 0.0025 (1/400) IU, kills 50% of the mice (white CD-1 mice, 2 to 3 months old, weighing 18 to 20 g) 4.5 days after injection. Lyophilized and purified tetanus toxin, a gift from Sclavo Laboratories (Siena, Italy), titrated in mice and containing 200,000 minimal lethal doses per

ml was used. The toxin was dissolved in 1% peptone-buffered solution (pH 7.4) and diluted immediately before use.

Titration of tetanus antitoxin was carried out by the method of Ipsen, by which the antitoxin titer is calculated on the basis of the time of death of the mice (8). The sera were diluted in 1% peptone-buffered solution (pH 7.4), mixed with toxin, and incubated at 20°C for 1 h; 0.5 ml of each mixture was injected subcutaneously in the lower back of CD-1 mice. One mouse was used for each serum dilution. The international standard for tetanus antitoxin was used as a reference.

CIE. The CIE technique described by Winsnes (20) was used. Briefly, glass plates (9.2 by 8.4 cm) were coated with a 0.1-cm layer of 1% agarose-M (LKB, Bromma, Sweden) in a barbital-calcium lactate buffer (pH 8.6; ionic strength, 0.065). Double rows of opposing wells, 4 mm in diameter and 8 mm apart (from center to center), were punched lengthwise into the gel. A slide of this size accommodated three double rows, with nine paired wells in each row. Thus, 27 specimens could be analyzed simultaneously on one slide. Serum samples (10 μ l per well) were placed in the rows of wells near the anode.

Electrophoresis was carried out for 60 min before tetanus toxoid (10 μ l per well, 5.5 fl/ml in 0.9% saline) was added to the rows nearest the cathode, after which electrophoresis was continued for a further 30 min. Electrophoresis was run at 6 V/cm, using a water-cooled apparatus and the barbital-calcium lactate buffer. The agar plates were allowed to stand in a moist chamber overnight before being read. Dilutions of the international standard for tetanus antitoxin were used as the reference serum, and the antitoxin content of unknown sera was expressed in international units per milliliter.

ELISA. The ELISA described by Stiffler-Rosenberg and Fey (19) was used. The test was carried out as follows. (i) Disposable polystyrene cuvettes (LKB) were sensitized with purified tetanus toxoid (1.65 fl/ml) diluted in a 0.1 M carbonate-bicarbonate buffer (pH 9.6) for 4 days at 4°C. The cuvettes were then washed three times for 3 min each in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (E. Merck AG, Schuchardt, Germany) and shaken dry. (ii) Serum samples were diluted in PBS-Tween 20-bovine serum albumin (BSA; final concentration of BSA, 0.5%). One milliliter of the diluted serum was added to each cuvette, and the cuvettes were incubated for 1 h at 37°C and then overnight at 4°C. The cuvettes were washed as before in PBS-Tween 20 and shaken dry. (iii) Rabbit immunoglobulin G horseradish peroxidase antihuman globulin (Cappel Laboratories, Downingtown, Pa.) was diluted in PBS-Tween 20-BSA (final concentration of BSA, 2%). One milliliter of the diluted antiglobulin conjugate was added to each cuvette, and the cuvettes were incubated for 1 h at 37°C then washed again and shaken dry. (iv) 5-Aminosalicylic acid (Sigma Chemical Co., St. Louis, Mo.) diluted in distilled water at a concentration of 80 mg/100 ml (pH 6) was used as an enzyme substrate. One volume of H₂O₂ at a concentration of 0.05% in distilled water was mixed with 9 volumes of the enzyme substrate solution. Immediately after preparation, 1 ml of this solution was added to each cuvette. The enzymic hydrolysis of the substrate was stopped after 1 h by the addition of 0.1 ml of 1 M NaOH. (v) The results are

expressed as the absorbance at 490 nm, determined in a spectrophotometer with a 1-cm light path.

The unknown sera were measured against the reference human antitoxin serum (Tetabulin, 250 IU/ml; Immuno, Vienna, Austria), and the antitoxin content was expressed in international units per milliliter.

Statistical analysis. The regression equations were calculated by the method of least squares (4).

RESULTS

Correlation of antitetanus antibody titers obtained by TRBC-HA and NT. To investigate whether the tetanus antibodies detected by the TRBC-HA assay could be responsible for the neutralization of the tetanus toxin in mice, we calculated the correlation between the TRBC-HA and NT titers in sera from 97 persons of both sexes and different ages. The correlation between the two assays was very good ($r = 0.851$) (Fig. 1). The results obtained also show that TRBC-HA antibody titers of 0.25 and 0.5 HU/ml correspond to antitoxin antibody values ranging from 0.003 to 0.72 IU/ml and from 0.1 to 1.24 IU/ml, respectively. Titers of 1 HU/ml determined by the TRBC-HA assay correspond to a

FIG. 1. Scatter diagram of the relationship between NT and TRBC-HA titers. The line represents the regression equation between the variables, calculated by the method of least squares.

TABLE 1. Comparison of antitetanus antibody levels as determined by TRBC-HA, CIE, and ELISA tests in 55 normal sera

No. of serum samples	Antibody level		
	TRBC-HA (IU/ml)	ELISA (IU/ml $\pm$ SD) ^a	CIE (no. positive/total)
6	0.062	0.024 $\pm$ 0.021	0/6
7	0.125	0.093 $\pm$ 0.074	0/7
8	0.25	0.132 $\pm$ 0.078	0/8
5	0.5	0.225 $\pm$ 0.162	0/5
8	1	0.312 $\pm$ 0.163	0/8
6	2	0.79 $\pm$ 0.64	2/6
5	4	0.91 $\pm$ 0.58	4/5
10	8	2 $\pm$ 1.22	10/10

^a Values represent the arithmetic mean of the tested samples.

range of antitoxin antibodies from 0.97 to 3.7 IU/ml.

According to different authors (5, 7, 9, 13, 14), 0.1 IU/ml can be considered a safe level of protection against tetanus infection. We found that a TRBC-HA value of 0.5 HU/ml corresponded to values ≥ 0.1 IU/ml in 100% of the samples tested. For this reason a level of 0.5 IU/ml as determined by the TRBC-HA assay permitted us to discriminate protected (≥ 0.1 IU/ml) from unprotected persons (< 0.1 IU/ml).

Comparison of the level of antitetanus antibodies determined by TRBC-HA, ELISA, and CIE assays. To compare the level of antitetanus antibodies detected by ELISA and CIE with those detected by the TRBC-HA assay, we choose 55 sera from normal persons and divided them on the basis of different TRBC-HA tetanus antibody titers. The results obtained (Table 1) show that the CIE assay was positive for two of the six samples with TRBC-HA values of 2 HU/ml. The CIE assay was 100% positive (10 of 10 samples) only for TRBC-HA titers of 8 HU/ml. Antitetanus antibody values as assayed by ELISA seemed to be in the same range as those determined by the TRBC-HA assay.

Comparison of TRBC-HA and ELISA antitetanus antibody titers. A large number of human sera (670 samples) were evaluated with the TRBC-HA and ELISA tests. The results obtained showed very good correlation ($r = 0.85$) between the titers obtained by these two techniques (Fig. 2). The results showed also that the TRBC-HA assay had a relatively greater sensitivity than the ELISA. In fact, at low TRBC-HA titers (0.007, 0.015, 0.031, and 0.062 HU/ml), the number of sera found to be negative by ELISA were 4 of 6, 20 of 38, 54 of 73, and 46 of 146, respectively.

However, in the crucial range from 0.125 to 1 HU/ml, the number of serum samples tested that had the same titers when evaluated with both techniques were 68 of 122 (0.125 HU/ml), 44 of

73 (0.25 HU/ml), 23 of 47 (0.5 HU/ml), and 22 of 49 (1 HU/ml) (Fig. 2).

DISCUSSION

We showed previously that the passive TRBC-HA test for the detection of antitetanus antibodies in healthy people offers a sensitivity that is 20-fold higher than the same method using sheep erythrocytes (M. Pitzurra et al., Bull. W.H.O., in press). The correlation obtained between HA and neutralization (Fig. 1) suggests that the same antibody may be being measured in each assay.

Furthermore, the results indicate that antibody titer levels of 0.5 HU/ml correspond to antitoxin antibody levels of ≥ 0.1 IU/ml. Although a level of antitoxin antibodies of 0.01 IU/ml is considered protective (12, 15–18), many authors believe that only a level of 0.1 IU/ml can confer a safe level of protection (5, 7, 14). It follows that, for clinical purposes, the level of

FIG. 2. Scatter diagram of the relationship between ELISA and TRBC-HA titers. The line represents the regression equation between the variables, calculated by the method of least squares.

0.5 HU/ml found with the TRBC-HA assay can be considered a discriminating point between protected and unprotected persons. The preliminary comparison of the CIE, ELISA, and TRBC-HA assays indicates that CIE has limited sensitivity in comparison with the other two tests. In fact, the CIE test begins to be positive only with sera having ≥ 2 HU/ml. For this reason, CIE can be considered useful only in screening sera containing a relatively high level of tetanus antitoxin (≥ 8 HU/ml) (20).

On the other hand, the ELISA has a range of sensitivity similar to that of the TRBC-HA assay for values between 0.125 and 1 HU/ml. The ELISA as performed by us is a macrotest and very wasteful of reagents. Such a procedure would have limited routine use. However, the ELISA performed as a microtest might be considered for the purpose of screening for tetanus immunity. In spite of this, the TRBC-HA test offers several advantages over ELISA. (i) TRBC-HA is a less time-consuming assay, requiring only 40 min instead of the 24 h needed for the ELISA; (ii) the TRBC sensitized with tetanus toxoid can be stored in lyophilized form for months without loss of sensitivity or reproducibility; and (iii) the TRBC-HA assay is less expensive than ELISA and requires no special equipment.

In conclusion, the TRBC-HA assay offers many advantages over the ELISA and CIE tests in all situations where the combination of low cost with rapidity and sensitivity in the determination of tetanus immunity levels is necessary (2, 3, 6).

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