Immunoenzymatic Assay of Anti-Diphtheric Toxin Antibodies in Human Serum

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An enzyme-linked immunosorbent assay was developed for measuring immunoglobulin G anti-diphtheric toxin antibodies in human serum. The assay was done in plastic plates coated with purified diphtheric toxoid. Since a straight-line relationship was found between logs of extinction values and of antibody concentrations, with a very constant slope, serum titers could be expressed as log10 of the serum dilution corresponding to a definite optical density, such as 0.5. The assay furnished highly reproducible titers on a continuous range, with coefficients of variation less than 10%. Only one or two serum dilutions were usually sufficient for serum titration. To establish correspondence of the enzyme-linked immunosorbent assay titers with biologically determined antitoxin international units, a regression equation was fitted between the respective values for 112 serum samples titrated in both tests. The enzyme-linked immunosorbent assay titer of 2.38 corresponded to an antitoxin titer of 0.01 U, which is considered as the minimal protective level. Simple to perform, economical, and precise, the immunoenzymatic assay seems to be a very practical procedure for seroepidemiological purposes.

Diphtheria is still a serious problem in many underdeveloped countries, with significant morbidity rates (8). Although the immunization program for children in Brazil follows international recommendations (7), high diphtheria morbidity rates still occur for children under 10 years of age (1, 2). For evaluation of immunization procedures and the vaccine itself, antibody levels against diphtheric toxoid are useful to show the immune status of a population. However, the biological assay of anti-diphtheric toxin antibodies in human serum is not practical for seroepidemiological purposes, regardless of its sensitivity and reproducibility. The hemagglutination test with sensitized erythrocytes with diphtheria toxoid or toxoid is easier to perform and of low cost; however, the assay is only semiquantitative, and titers are reported in steps of serum dilutions at intervals. More precise results might be obtained with an enzyme-linked immunosorbent assay (ELISA) that would define titers over a continuous range.

In this paper we present our results for the development and standardization of an ELISA for immunoglobulin G antibodies against diphtheric toxoid in human serum and for establishing correspondence between ELISA titers and biologically determined antitoxin units.

MATERIALS AND METHODS

Human sera. Venous blood was collected from first-grade school children 6 to 9 years old. Detailed registers on the doses of diphtheric vaccine were available. Samples were collected before a new dose of diphtheric vaccine and 30 to 60 days after (manuscript in preparation). Serum separated from clotted blood was stored at −20°C for a few days until tested.

Biological assay. Anti-diphtheric toxin antibodies were titrated by the rabbit intradermic test according to the Frazer and Wigham modification of the technique of Römner and Sames (3). For this purpose diphtheric toxoid (batch 74-6, Instituto Butantan, São Paulo, Brazil) was titrated (Lr) against 5 × 10−3 IU of the Anti-Diphtheric Standard Serum, International Laboratory of Biological Standards, Copenhagen Statens Serum Institut, Denmark. In this way an intradermal injection with 0.2 ml of the stabilized toxin-antitoxin mixture should produce a 10-mm-diameter, nonnecrotic, erythematous area after 48 h. For the assay, serum dilutions were incubated with the toxoid for 60 min at 37°C and 18 h at 4°C, and 0.2 ml of the mixture was intradermally inoculated in adult white rabbits. Readings were done after 48 h, with reference to the standard mixture inoculated at the same time.

ELISA. Polystyrene plates with U-shaped wells (Interlab, São Paulo, Brazil) were coated with purified diphtheric toxoid (2.500 LFL/ml; Connaught Laboratories, Ontario, Canada). To prepare the plates the toxoid was diluted in 0.06 M sodium carbonate buffer (pH 9.6), and 200-μl volumes were added to the wells and left overnight at 4°C. The following morning the plates were emptied and drained over filter paper, filled with 2% gelatin in carbonate buffer, and incubated for 2 h at 37°C. After being washed three times with PBST (0.15 M NaCl, 0.01 M phosphates [pH 7.2], 0.05% Tween 20), the plates were drained and used the same day.

For the test, 200-μl volumes of serum dilutions in PBST were added to the wells of sensitized plates, incubated at 37°C for 60 min, and washed three times in PBST. Two hundred microliters of peroxidase anti-human immunoglobulin G γ-chain-specific conjugate (prepared by the method of Wilson and Nakane [10]) diluted in PBST was added to each well, followed by incubation at 37°C for 60 min and washing of the plates. The plates were then left at room temperature with 200-μl volumes of a chromogen solution (10 mg of orthophenylenediamine and 10 μl of 30% hydrogen peroxide in 20 ml of citrate-phosphate buffer [pH 5.0]). The reaction was stopped after 30 min with 1 drop of 4 M H2SO4, and the
plates were directly read at 492 nm in a MicroELISA Minireader Photometer (Dynatech Laboratories, Inc., Alexandria, Va.) in optical densities.

To determine the optimal antigen concentration for high sensitivity and low background, the plates were coated with serial twofold dilutions of diphtheric toxoid starting at 1:25. Best results were obtained for the 1:400 antigen dilution. For maximal activity, the conjugate could be used at 1:2,500.

RESULTS

Serum titrations. When serum samples were assayed in twofold dilutions starting from 1:20, straight lines were obtained by plotting logarithms of optical densities and serum dilutions between extinction values of 0.2 and 1.8. When comparing such dilution curves from a series of serum samples, very similar slopes were seen (Fig. 1), for which a value of 0.73 was found to express the relationship between respective increases in log extinction and log dilution.

Serum titers were expressed as log₁₀ of the serum dilution, corresponding to an optical density of 0.5, which was chosen as a definite point midway in the straight part of the curve. In this way titers could be determined from one optical density value falling within this section of the curve, according to equation A: \[ T = \log D + (\log E - \log 0.5)/0.73, \] with \( E \) being the extinction value observed for serum dilution \( D \). Usually two serum dilutions were tested, and the titer was determined for each optical density between the extreme values of 0.2 and 1.8, a medium value being taken as the serum titer. In this case the titer could be alternatively determined according to equation B: \[ T = \log D_1 + (\log E_1 - \log 0.5)(\log D_2 - \log D_1)/(\log E_1 - \log E_2), \] with \( E_1 \) and \( E_2 \) being the extinction values for dilutions \( D_1 \) and \( D_2 \). No significant differences could be found between the titers determined by both procedures. Table 1 shows the results for 22 serum samples, with the \( t \) test indicating the differences as not significant \( (t = 0.0257) \).

To avoid the influence on titers of daily variations in test sensitivity, a reactive reference serum sample was run in each test, and serum titers were corrected according to observed variations in the reference serum titer. For this serum sample, from titer determinations on 21 different occasions the mean ± standard deviation was 3.45 ± 0.24 with a coefficient of variation of 7.07%. A second standard serum sample, which was also titrated at the same time, had a mean ± standard deviation titer of 2.68 ± 0.23 with a coefficient of variation of 8.54%. When correcting titers of this serum sample according to variations in the reference serum titer, the mean ± standard deviation titer was 2.56 ± 0.12 with a coefficient of variation of 4.49%. Variances for noncorrected and corrected titers were, respectively, 0.0524 and 0.0133, with a quotient of \( F = 3.94 \), which is significant at the 1% level.

Titer reproducibility. To evaluate inter-test reproducibility of titers, duplicates of 27 serum samples were titrated on different days. There was no significant difference in the duplicate titers, for which a variance of 0.4067 was found, with a standard deviation of 0.20167 and a coefficient of variation of 7.1%.

Correspondence between ELISA and antitoxin units. Comparison between ELISA titers and biologically determined antitoxin units in isolated serum samples showed great variation. To establish correspondence of ELISA titers to antitoxin units, 112 serum samples were assayed in both tests (Fig. 2). Taking antitoxin units as a reference (independent variable \( x \)), a series of regression equations were then adjusted by minimum squares. The determination coefficient (\( R \) square) and \( F \) values (for 1.110 degrees of freedom) were, respectively, 0.257 and 38.0 for linear, 0.170 and 22.5 for exponential, 0.636 and 192.1 for power, and 0.658 and 211.7 for logarithmic regression. The best fitting equation was therefore \( \tilde{y} = 4.123 + 0.8779 \log x \).

According to this equation, an antitoxin titer of 0.01 U, considered as the minimal protective antibody level, corresponded to an ELISA titer of 2.38. For such cutoff values, agreement as to protection or nonprotection was seen for 101 (90.2%) of the 112 serum samples titrated by both methods.
This test determining for the titers in several cases (4.3%; 95% confidence limits, 95% [0.9 to 12.2%]).

DISCUSSION

The ELISA for quantitation of anti-diphtheric toxin antibodies described in this paper should be a valuable tool for determining immunological protection against diphtheria. This test should be especially useful for population studies, since it is economical and practical for large routine purposes. When compared with the hemagglutination test, it presents several advantages, such as the possibility of precise results through the assay of only one or two serum dilutions. Because of the linear relationship between logs of extinction values and of antibody concentrations, and the constant slope observed, serum titers could be expressed as the serum dilution corresponding to a definite optical density value. This was arbitrarily taken as 0.5. Assaying the 1:200 serum dilution was sufficient to define antibody levels corresponding to immunological protection.

The ELISA was very reproducible, with coefficients of variation of titers less than 10%. It should be noted that in the ELISA titers were obtained in a continuous range, in contrast to the twofold dilution step values at intervals which are given by the hemagglutination test. Correction of titers for daily variations in test sensitivity, as indicated by the titer variations of a reference serum, also contributed to titer reproducibility.

Correspondence of ELISA titers to biologically determined antitoxin units could not be established from the assay of isolated serum samples taken as references. Large variations were found between different preparations taken as standards. At the same time, when a large number of serum samples were titrated by both assays, scattering of ELISA titers was observed for each antitoxin value. Such scattering of ELISA values, in comparison with other assays based on stepwise titration methods, has been observed in several other complex antigen-antibody systems, for cytomegalovirus (4), rubella (6), Toxoplasma gondii (5), and Entamoeba histolytica (9).

Thus, correspondence between ELISA titers and antitoxin units was determined by fitting the best regression equation between values from 112 serum samples titrated in both tests and taking the antitoxin units as a reference. We established that the 2.38 ELISA titer correlated to an antitoxin titer of 0.01 U, which is considered as the minimal protective level. For these cutoff titers 90% agreement was found between both assays in discriminating protected from nonprotected individuals, the correspondence being expressed also by the high copositivity and conegativity indices of the ELISA as they referred to the biological assay. Antibodies detected by both techniques may not be the same. Perhaps better agreement between both assays could result from performing the ELISA with a toxoid antigenic fraction that might be more closely related to protective antibodies. Otherwise, observed differences may just be the expression of poorer reproducibility of the biological assay, the results of which could be only a more or less accurate approximation of the unknown real protection values.

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LITERATURE CITED