

## Rapid Quantitative Microenzyme-Linked Immunosorbent Assay for Tetanus Antibodies

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A rapid quantitative microenzyme-linked immunosorbent assay for the detection of immunoglobulin G antibodies to tetanus toxoid is described. The assay is performed in 2.5 h and detects antibody levels of between 0.1 and 6 U/ml. Quantitation of the antibody concentration is unique in that multiple serum dilutions are tested on a single patient, the results are plotted as an antibody dilution curve, and the area under the curve is integrated for a final concentration. Tests for immune status on sera from 77 individuals demonstrated tetanus antibody concentrations of  $\geq 0.1$  U/ml in 92% of a pediatric group and 62% of an adult group. The microenzyme-linked immunosorbent assay procedure is reproducible and is useful for the determination of immune status and immunization response.

Measurement of immunity to clinical tetanus has traditionally been based on the toxin neutralization test developed by von Behring and Kitasato in 1890 and more recently described by Barile et al. (1). However, this assay requires the use of animals and takes several days to perform. Several newer methods have been adapted to measure antibodies to tetanus toxoid, including radioimmunoassay (8, 10, 13), a modified radioimmunoassay in which protein A is used (7), latex agglutination (2), and enzyme-linked immunosorbent assay (ELISA) (10, 14).

The original ELISA methodology was independently developed and reported by Engvall and Perlmann (6) and Van Weeman and Schuur (15) in 1971. Stiffler-Rosenberg and Fey (14) described a tetanus antibody ELISA which was performed in test tubes and had two overnight incubation periods. Layton (10) developed a micro-ELISA method for detecting tetanus antibodies and compared the results with those of the radioimmunosorbent technique. This ELISA procedure was based on the results of a single serum dilution and employed relatively long incubation steps. However, the use of a single serum dilution for quantitation poses problems, as pointed out by Bullock and Walls (3) and discussed recently by de Savigny and Voller (5).

We report a rapid quantitative micro-ELISA method for the detection of antibodies to tetanus toxoid and propose a new method of quantitation based on integration of the area under the antibody dilution curve, which is constructed by measuring the amount of antibody in each of six dilutions of the test serum or standard antitoxin.

We describe the antibody response to tetanus vaccination in normal individuals and in a group of children being evaluated for possible immune deficiencies, and the immune status in the general population.

### MATERIALS AND METHODS

**Antigen.** Tetanus toxoid refined concentrate (lot 013076, 500 limit flocculation units per ml) was obtained from Wyeth Laboratories, Inc., Marietta, Pa. (purity, 769 Lf/mg of protein nitrogen; protein nitrogen, 0.65 mg/ml).

**Sera.** The human serum (HS) pool was prepared by combining serum samples from pilot tubes obtained from healthy donors at the American Red Cross Blood Services, Connecticut Region, Farmington, Conn.

Tetanus immune globulin (TIG) (human; Hyper-Tet; 250 U/1.0 ml) was obtained from Cutter Laboratories, Berkeley, Calif.

Sera selected from samples submitted to the clinical laboratory from 77 individuals, 27 children and 50 adults, were used to evaluate the procedure. Sera were also obtained at the Employee-Student Health Clinic from individuals before and at intervals after the administration of tetanus-diphtheria toxoids (adsorbed). Multiple serum samples were collected from 12 pediatric patients who were being evaluated in a pediatric immunology clinic for possible immune deficiencies.

**Conjugate.** Horseradish peroxidase conjugated to goat anti-human immunoglobulin G (Fc fragment; lot 14925) was purchased from Cappel Laboratories, Inc., Cochranville, Pa.

**Buffers.** Carbonate buffer, pH 9.6 (coating buffer), was prepared by adding 1.59 g of  $\text{Na}_2\text{CO}_3$ , 2.93 g of  $\text{NaHCO}_3$ , and 0.2 g of  $\text{NaN}_3$  to 1 liter of distilled water.

Phosphate-buffered saline (pH 7.2) with 0.05% Tween 20 (PBST) was used for serum and conjugate dilutions and in washing procedures.

Citrate buffer (pH 4.0) was prepared by mixing 66 ml of 0.1 M citric acid, 34 ml of 0.1 M sodium citrate, and 100 ml of distilled water.

**Substrate-chromogen.** ABTS [2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate)] was purchased from Boehringer Mannheim Corp., Indianapolis, Ind. A stock solution was prepared by adding 45 mg of ABTS to 1 ml of distilled water. The working solution of substrate-chromogen consisted of 150 µl of ABTS stock solution, 10 ml of citrate buffer (pH 4.0), and 40 µl of 3% hydrogen peroxide.

**Microtiter plates.** Linbro enzyme immunoassay microtiteration plates (96-well, flat-bottomed [catalogue no. 76-381-04, no. 8] plates; Flow Laboratories, Inc., McLean, Va.) were used for the ELISA procedure.

**ELISA procedure.** This rapid ELISA, in which a 1-h coating step, 30-min serum incubation, 30-min conjugate incubation, and 5- to 10-min substrate reaction are used, was performed as follows. Tetanus toxoid was diluted to 1:1,000 in carbonate buffer (pH 9.6), and 50 µl was dispensed into wells. The outer wells of the plate were not used because of reports of inconsistent results. The plate was tightly covered and incubated for 1 h at 37°C. After coating with toxoid, the microtiter plate was washed four times with PBST and blotted to remove excess buffer. Serial twofold dilutions (1:10 through 1:320) were prepared in PBST from both the HS pool and serum specimens. Fifty microliters each of the HS pool and serum specimen dilutions were dispensed into duplicate wells. The plate was covered, incubated for 30 min at 37°C and then washed four times with PBST and blotted. Fifty microliters of a 1:200 dilution of conjugate was added to all wells, and the plate was covered and incubated for 30 min at 37°C and again washed four times. Substrate-chromogen working solution was prepared and 100 µl was dispensed into wells. After 4 to 5 min of incubation at room temperature, optical density (OD) was measured for the HS pool 1:10 dilution. When the OD was between 1.2 and 1.5, all OD values were recorded by an Automatic Titertek Multiskan ELISA plate reader (Flow Laboratories) at 414 nm. Controls included wells without serum sample (background) and non-coated wells.

**Calculations.** To illustrate the method used to calculate the area under the curve, results of a sample tetanus ELISA test are presented. Table 1 shows the duplicate OD values for the HS pool, a serum sample, and 12 background replicates. The curves produced by the average OD determinations of the serum dilutions from which background has been subtracted are shown in Fig. 1. The area under the dose-response curve was calculated by the midpoint estimate procedure for approximating the integral function. The general formula is:

$$\sum^n f(x_i) \Delta x$$

where  $i = 1$ . The working formula is:

$$\text{Area} = \frac{X}{2r} \left[ 2(\sum \text{OD middle dilutions}) + (\sum \text{first} + \text{last dilutions}) - \frac{4(n-1)(\sum \text{OD background})}{q} \right]$$

TABLE 1. Results of OD determinations for tetanus antibody (ELISA) in the HS pool and a representative patient (patient 6)

Serum dilution	HS pool OD for replicate:		Serum sample OD for replicate:		OD background (n = 12) <sup>a</sup>
	1	2	1	2	
1/10	1.29	1.38	0.48	0.46	0.015 0.014 0.003
1/20	1.02	1.01	0.34	0.37	0.012 0.012
1/40	0.58	0.61	0.22	0.21	0.011 0.037
1/80	0.31	0.37	0.11	0.14	0.041 0.018
1/160	0.20	0.21	0.08	0.08	0.025 0.027
1/320	0.12	0.11	0.05	0.06	0.032
					Σ = 0.247

<sup>a</sup> Number of individual background determinations.

where  $X$  is the distance between points on the  $X$  axis (for this example, an orbit ray value of 20 was used),  $r$  is the number of replicates of each dilution point,  $n$  is the number of dilutions tested, and  $q$  is the number of background replicates.

The calculated area of the test serum sample shown in Fig. 1 is 18.68, and that of the HS pool is 55.52. To correct for daily variation, the area under the curve of the HS pool is corrected to 100%. This correction factor is applied to each of the patient samples. In this example, the final corrected ELISA area is 34.

**TIG standard curve.** Dilutions of TIG (250 U/ml) were prepared in PBST so that the final concentrations were 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, and 0.05 U/ml. Each of these globulin preparations was then treated as a single sample, and serial twofold dilutions were made from 1:10 through 1:320. The ELISA procedure was performed on these samples, the area under the curve was calculated, and the results were expressed as a percentage of the HS pool internal control.

### RESULTS

To evaluate reproducibility, one serum sample was tested by ELISA 17 times on 5 separate days and in different positions on the microtiter plate. The mean of the 17 tests was 104.1, the range was 84 to 120, 1 standard deviation was 10.5, and the coefficient of variation was 10.1%.

Figure 2 shows the standard curve for TIG expressed in units per milliliter as a function of

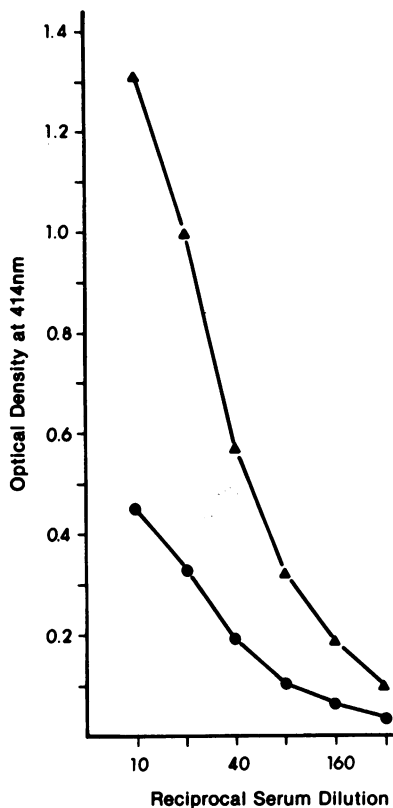


FIG. 1. Representative antibody curves (ELISA) of the human serum pool and patient 6 corrected for background OD. ▲, HS pool; ●, serum sample (patient 6).

the area under the curve. The data was plotted as a log-log plot. The points on the curve represent four separate analyses performed on different days. Linear regression analysis indicated that the correlation coefficient was 0.981 (standard deviation,  $\pm 0.120$ ), the y axis intercept was 0.013, and the slope was  $1.205 \pm 0.046$ . The lower limit of detection was 0.1 U/ml. The datum points show the range of determinations for a particular concentration of TIG. The log-log plot approximates a straight line. This standard curve was used to convert ELISA area to antibody units per milliliter.

Figure 3 shows the results of the ELISA test for tetanus antibodies when 77 serum samples from 50 adults and 27 children were analyzed. Using the TIG standard curve, ELISA areas were converted to units per milliliter. In Fig. 3, numbers of individuals (children aged 2 to 16 years and adults aged  $\geq 17$  years) are shown as a function of tetanus antibody in units per milliliter. There were two distinct populations observed: one which had either no or barely detectable antibody ( $< 0.1$  U/ml), and a second

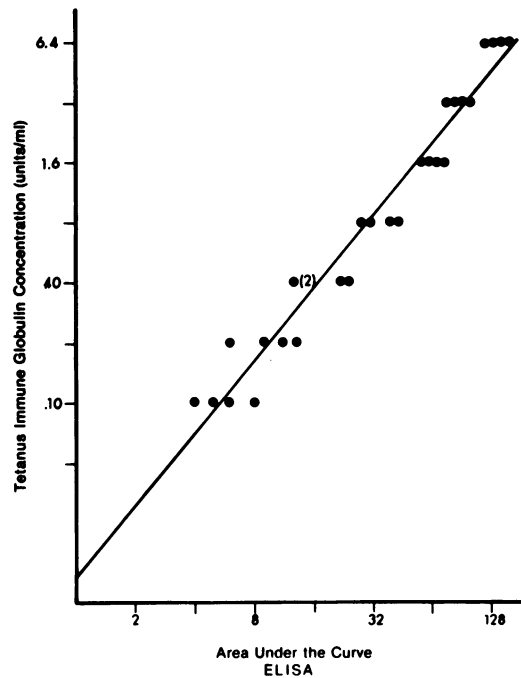


FIG. 2. Standard curve of tetanus immune globulin showing calculated areas under the curve as a function of globulin concentration (units per milliliter). Points on the curve were generated from four separate ELISA analyses.

which had measurable antibody levels ( $\geq 0.1$  U/ml). The population with antibody levels of  $< 0.1$  U/ml comprised 7 and 38% of the sera from children and adults, respectively, whereas the population with levels of  $\geq 0.1$  U/ml comprised

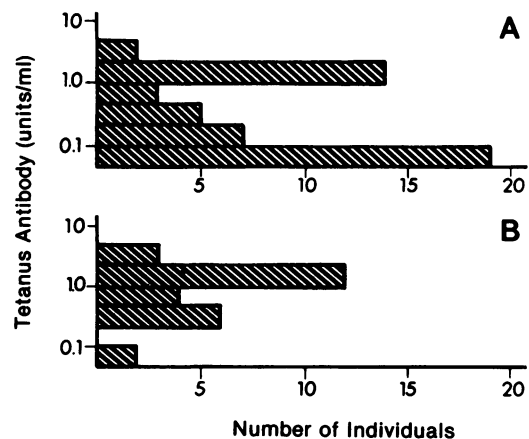


FIG. 3. Tetanus antibody (ELISA) results for 77 serum samples from 50 adults (A) and 27 children (B). Areas under the curve were calculated from ELISA tests and converted, using the TIG standard curve, to corresponding units per milliliter.

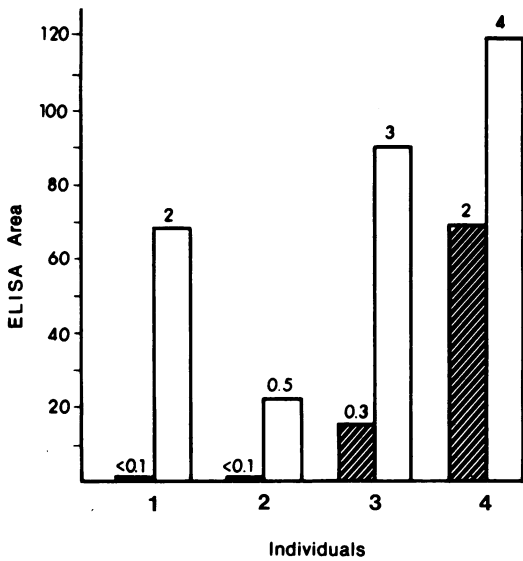


FIG. 4. Quantitation of the tetanus antibody level (ELISA) of four normal individuals before and after immunization. Antibody levels before (▨) and after (□) tetanus toxoid administration are shown. Numbers above the bars indicate the tetanus antibody levels (in units per milliliter).

93 and 62% of the sera from children and adults, respectively.

Results of the ELISA test for tetanus antibodies performed on sera collected from four patients before and after tetanus toxoid immuniza-

tion are shown in Fig. 4. Two patients, 1 and 2, had no history of tetanus immunization, and sera from both lacked detectable tetanus antibody [ELISA area = 0 (<math><0.1</math> U/ml)] when tested before toxoid administration. The remaining two patients, 3 and 4, had prior histories of tetanus immunization and, when first tested in this study, showed ELISA areas of 15 (0.3 U/ml) and 69 (2 U/ml), respectively. Patients 1 and 2 received two toxoid injections and patients 3 and 4 received one toxoid injection between pre- and posttesting. ELISA areas increased from 0 to 67, 0 to 22, 15 to 90, and 69 to 119 for patients 1, 2, 3, and 4, respectively (Fig. 4). When these changes in tetanus antibody are expressed in units per milliliter, it can be seen that the values for patients 1, 2, 3, and 4 increased from <math><0.1</math> to 2, <math><0.1</math> to 0.5, 0.3 to 3, and 2 to 4 U/ml, respectively.

Figure 5 shows the ELISA test results for multiple serum samples collected from 12 pediatric patients. These children were being evaluated for possible immune deficiencies in a pediatric immunology clinic. Six patients had low or undetectable serum antibody levels to tetanus toxoid, despite a history of prior immunization. These children were given one to two doses of tetanus toxoid as a booster to evaluate their B cell functions in the production of specific antibody upon antigen challenge. All six patients produced significant tetanus antibody after these booster immunizations. These and other studies were helpful in ruling out an underlying B cell immune deficiency disorder.

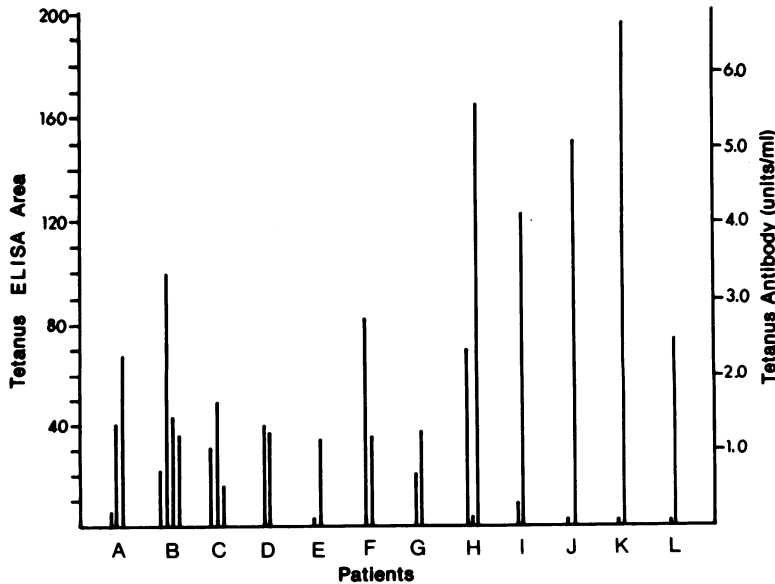


FIG. 5. Quantitation of the tetanus antibody level (ELISA) of 12 immunology clinic patients before and after challenge immunization.

## DISCUSSION

A variety of serological tests have been used to measure antibody to tetanus toxoid, and the ELISA has recently been added to the list. Stiffler-Rosenberg and Fey (14) describe a tetanus antibody ELISA procedure in which are used 1-ml volumes in test tubes, three serum dilutions, two overnight incubation steps, and alkaline-phosphatase as the enzyme. In an effort to make the test more economical, Layton (10) developed a tetanus ELISA assay in which smaller volumes in microtiter plates and the less expensive enzyme horseradish peroxidase are used. One serum dilution (1:10) and one overnight incubation step were used.

Bullock and Walls (3), in their study of the variables in the *Toxoplasma* antibody ELISA test, found that short incubation periods (total, 5 h) were satisfactory, and because their reactivity curves showed an extremely steep slope at the endpoint, they concluded that measurement of a single dilution of test serum was inadequate for quantitative tests. Another important consideration is that the antibody affinities of various sera differ, producing many possible dose-response curves, some of which are nonparallel (5).

The rapid micro-ELISA described in this paper is economical in terms of time (2.5 h of incubation) and reagents and introduces a new method of quantitating tetanus toxoid antibody. In early testing of this procedure, curves produced by plotting the OD readings of several dilutions of sera from various individuals were not always parallel. This result may be explained by the complexity of antibody responses by the individuals to multiple antigenic determinants in the tetanus toxoid structure (11) and to differences in antibody affinities (4). No single dilution of serum could be relied upon to accurately quantitate antibody levels. For example, in Fig. 6 the curves generated by plotting the ELISA results of a patient sample and the HS pool are not parallel; in fact, the two curves intersect between the 1:40 and 1:80 dilution. If the antibody from a patient is determined as a percentage of the HS pool for each of the serum dilutions (1:10 to 1:320), the values are 52, 66, 85, 125, 157, and 221% of the HS pool. Therefore, the most accurate and reproducible way to quantitate the antibody level of the patient is by integrating the sum total of the area under the experimentally plotted dilution curve and expressing it as a percentage of the total area of the HS pool internal control. When this calculation was performed for the sample (patient 5) in Fig. 5, the area was equal to 79%. Although this one patient sample (patient 5) reflected an extreme instance of discordance, many samples showed

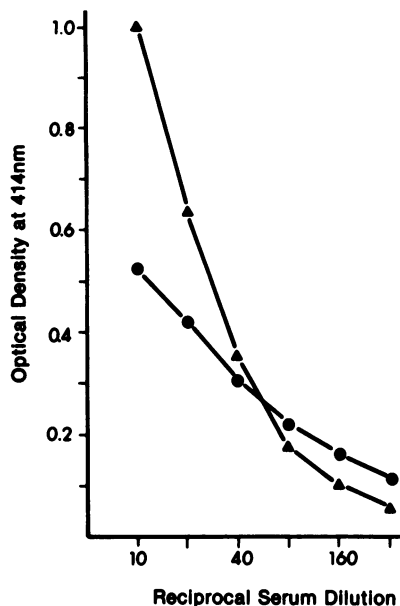


FIG. 6. Nonparallel antibody curves (ELISA) of a patient (patient 5) versus the HS pool. ▲, HS pool; ●, serum sample (patient 5).

this non-parallelism, although to a lesser degree.

The standard curve for the TIG relates the TIG concentration, in units per milliliter, as a function of the area under the curve. A doubling of the amount of TIG approximately doubles the area under the curve. This is not an exact 1:1 relationship, as the slope of the curve is 1.2, not 1.0. When plotted, however, the curve is linear in the range of concentrations tested. It should be noted that the assay probably cannot distinguish antibody concentrations of less than 0.05 U/ml because of the inherent background activity of the ELISA test.

It is generally considered that 0.01 U of tetanus antitoxin per ml is the minimum protective level, although some authors feel that 0.1 U/ml is a safe level (12). This value is predicated on a study in which it was noted that, of those individuals having levels of 0.1 U/ml on initial testing, 95% still showed tetanus antibody concentrations of at least 0.01 U/ml 5 to 6 years later. Conversely, children who at the first assay had levels of <0.1 U/ml risked having <0.01 U/ml 5 to 6 years later. The authors implied that 0.1 U/ml provides good protection and is a safe level of tetanus antibody (12). On the basis of results of the TIG standard curve, it appears that this ELISA procedure has the capability of determining a safe level of tetanus antibody.

Tests for immune status on 77 individuals showed that only 8% of the children, as compared with 38% of the adults, had tetanus

ELISA areas that corresponded to levels of <0.1 U/ml. This result may be expected, however, because most of these children had received their last tetanus booster within the previous 10 years. The range of ages in the adult group was between 17 and 85 years; 26% of the adults were >50 years old. Although the tetanus immunization histories of these adults are not available, it is reasonable to assume that many of them may not have had a tetanus booster injection within the last 10 years (the current recommended interval between tetanus boosters) (9).

The rapid quantitative micro-ELISA described is reproducible and is useful for determining immune status, assessing the response of an individual to tetanus toxoid immunization, and evaluating B cell dysfunction in children.

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