

Detection of Tetanus Antitoxin Using Eu^{3+} -Labeled Anti-Human Immunoglobulin G Monoclonal Antibodies in a Time-Resolved Fluorescence Immunoassay

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J Clin Microbiol 29, 1504-1507, 1991

Summary

Tetanus antitoxin in human sera was detected with solid-phase immunoassays in microtitration modules coated with tetanus toxoid by using Eu^{3+} -labeled anti-human monoclonal antibodies on the basis of an exactly calibrated antibody standard. The use of a time-resolved fluorescence immunoassay (TR-FIA) significantly improved the quantitative detection of tetanus antitoxin over that of the enzyme-linked immunosorbent assay (ELISA) technique because of its high sensitivity and its wide measurement range, detecting antibody levels between 0.001 and 12.5 IU/ml with a single serum dilution of 1:100. For the same purpose, two different serum dilutions (1:100 and 1:1,000) were needed in the ELISA technique.

TR-FIA is reproducible and can be performed in 3.5 hours. A study of 2,630 serum samples was undertaken to examine the age-dependent distribution of titer levels, indicating the decline of sufficient protection in patients older than 60 years. The wide measurement range of TR-FIA enabled fast examination of large numbers of serum samples without the need for repetition, with further sample dilution, as was often necessary in the ELISA procedure.

Introduction

Tetanus can be prevented effectively by prophylactic immunization with tetanus toxoid. The assay of tetanus antitoxin in human sera is frequently required in antibody survey and investigation of immunodeficiency. The minimum level to be sufficient for protection is thought to be 0.01 IU/ml (12). Case reports have shown that the recommended protection level is not sufficient to prevent tetanus (15). Therefore, a higher protection level must be attained. For this reason, a 10-fold-higher tetanus toxoid level is proposed (14,19). The true value of protection is not exactly known in human beings because all experiments and results are based on tests in mice. Differences in the individual reaction characteristics in cases of tetanus can, therefore, only be postulated. For these reasons, we suggest that 0.1 IU/ml provides good protection and represents a safe level of tetanus antibody. Since many men and women have satisfactory antitoxin levels, we propose that the antitoxin level be determined before revaccination to avoid possible hyperergic reactions.

Measurement of immunity to tetanus has traditionally been based on the toxin neutralization test. Several methods have been adopted to measure the amount of antibodies to tetanus

toxoid, including radioimmunoassay (6, 7, 11, 22), latex agglutination (1), and enzyme-linked immunosorbent assay (ELISA) (2, 3, 5, 6, 11, 18, 19, 23). Various solid-phase immunoassays have found increasing use in recent years in the determination of bacterial antibodies in human sera. Until now, the detection of tetanus antitoxin was carried out in our laboratory with the widely applied ELISA technique (17).

In search of an alternative method to the ELISA, we have developed a nonisotopic immunoassay based on the labeling of anti-human-immunoglobulin (IG) G monoclonal antibodies with Eu^{3+} . The amount of Eu^{3+} -labeled antibodies corresponds to the quantity of tetanus antitoxin. The principles of time-resolved fluorescence immunoassays (TR-FIA) were described earlier (9, 10) and have already been successfully used in the field of virology (8) in the measurement of hepatitis B surface antigen (20), antirubella antibodies (13), and hormones (16).

The present results demonstrate that the high sensitivity and the wide measurement range of TR-FIA make this test a useful tool in the quantitative detection of tetanus antibody levels. Thus, the measurement of both very low and very high antibody levels is facilitated, as proven under routine conditions in a study on age-dependent distribution of tetanus antitoxin in 2,630 men.

Materials and Methods

Sera. Ninety serum samples used to evaluate the procedure were collected from patients between 1 and 90 years of age. These samples had been submitted by the military hospital in Koblenz in order to determine the immune status of the patients before vaccination. The other 2,630 serum samples in our study were obtained from various hospitals in the Federal Republic of Germany.

Antigen. Tetanus toxoid of high purity (1,000 limit flocculation units per ml, 1,510 limit flocculation units of protein nitrogen per ml, and 4.20 g of protein per liter) was obtained from Behringwerke, Marburg, Federal Republic of Germany.

Antitoxin. Tetagam as the reference antitoxin was supplied by Behringwerke. The amount of antitoxin of the calibrated Tetagam (290 IU/ml) was ascertained in the toxin neutralization test in mice on the basis of a standard preparation under application of L+/10-dosis.

Conjugate. Eu^{3+} -labeled mouse anti-human IgG monoclonal antibody was obtained from Pharmacia LKB, Freiburg, Federal Republic of Germany. Eu^{3+} -labeled anti-human monoclonal antibody was used at a concentration of 25 $\mu\text{g}/\text{ml}$. Alkaline phosphatase-labeled goat anti-human IgG was purchased from Dianova, Hamburg, Federal Republic of Germany.

Standard laboratory diagnosis. Serological diagnosis of tetanus antitoxin was performed by the ELISA method routinely used in our laboratory as described previously (17). Briefly, high-binding, cobalt-irradiated polystyrene microwell modules were coated with the purified tetanus toxoid at a dilution of 1:6,400. Plates were incubated for 45 min with patient sera at a dilution of 1:1,000 (measuring range, 0.3 to 6.3 IU/ml) or 1:100 (measuring range, 0.03 to 0.6 IU/ml). Anti-IgG antibodies conjugated with alkaline phosphatase served as second antibodies. This incubation period (45 min) was followed by a reaction with the substrate (*p*-nitrophenylphosphate; Merck, Darmstadt, Federal Republic of Germany) for 20 min. All reagents were used in standard volumes of 100 μl , and all reactions were carried out at 37°C in a water

bath. The optical density at 405 nm was measured after the addition to each well of 50 μ l of stop solution (1 M NaOH) with a Titertek multiscan ELISA reader (Flow, Meckenheim, Federal Republic of Germany). The amount of tetanus antitoxin was calculated by linear regression. Sera were assayed in duplicate.

Experimental design. All experiments were randomly designed in microtiter strips. Each microtiter plate contained the sera of the standard curve and all patient sera as duplicates, positive control sera (Tetagam) as well as negative controls. The degree of correlation between ELISA and TR-FIA was assessed by a standard linear regression technique. Intra-assay and interassay studies were performed by using serum samples of high, medium, and low antitoxin levels as well as a reference Standard. Intra-assay and interassay experiments were done in replicates of 14 and 17 runs, respectively.

TR-FIA procedure. A comparison of the TR-FIA with the ELISA for the detection of anti-tetanus antibodies is shown in Table 1. Polystyrene microtitration modules were coated with the tetanus toxoid antigen (1:6,400), 200 μ l per well, in 0.06 M carbonate buffer (pH 9.6) by incubation for 4 h at room temperature, followed by blocking with 0.5% bovine serum albumin (BSA) in Tris buffer (0.05 M Tris HCl, 0.9% NaCl, 0.05% NaN₃ [pH 7.8]). Washing with Tris buffer containing 0.05% Tween 40 and incubation procedures with the assay buffer (0.05 M Tris [pH 7.8] containing 0.5% BSA, 0.05% NaN₃, and 0.02% Tween 40) were carried out at room temperature. Dilutions of the sera, standards for calibration, and control sera were prepared as duplicates in assay buffer (250 μ l per well) and incubated for 2 h. Solid-phase-bound antigen-antibody complexes were incubated with Eu³⁺-labeled anti-human IgG monoclonal antibody (200 μ l per well) for 1 h, followed by washing. After the final incubation and removal of the excess Eu³⁺-labeled antibodies, the amount of bound Eu³⁺ was released and transferred into a fluorescent chelate by adding TR-FIA enhancement solution (Pharmacia LKB), 200 μ l per well. After approximately 10 min, the fluorescence was measured with a single photon-counting fluorometer (Arcus Fluorometer, Pharmacia LKB). The results from sera and standards were calculated with logarithmic fitting to the response curve. Taking into account the precision and the correlation coefficient, response and dose were calculated on the basis of linear regression in order to minimize proportional deviation. The measurement range was 0.001 to 12.5 IU/ml within one single serum dilution.

TABLE 1. Comparison of the TR-FIA and ELISA systems for detection of tetanus antitoxin

Step	Detection system characteristic ^a	
	TR-FIA	ELISA
1. Target	Tetanus toxoid, 1:6,400 dilution	Tetanus toxoid, 1:6,400 dilution
2. Probe	First antibody; sera, 1:100 dilution	First antibody; sera, 1:1,000 ^b or 1:100 dilution
3. Detection	Second antibody, Eu ³⁺ -labeled MAb; addition of enhancement solution	Second antibody, alkaline phosphatase-labeled PAb; reaction with substrate
4. Signal	Fluorescence	Color
5. Calculation of amt of antitoxin	Range of detection, 0.001–12.5 IU/ml	Range of detection, 0.03–6.3 IU/ml

^a MAb, monoclonal antibody; PAb, polyclonal antibody.

^b Typical dilution for routine use.

Results

The time-resolved immunofluorescence technique revealed good and reproducible results. A typical standard curve obtained in tetanus antitoxin TR-FIA is shown in Fig. 1. The linear regression analysis indicated that the correlation coefficient was 0.993 ($y = 1.130x + 11.667$). With a final assay dilution of 1:100, the measurement range was at least 0.001 to 12.5 IU/ml. In the case of the ELISA, two different sample dilutions (1:100 and 1:1,000) were needed to

cover the same tetanus antitoxin range. Thus, TR-FIA can be regarded as a rapid immunoassay.

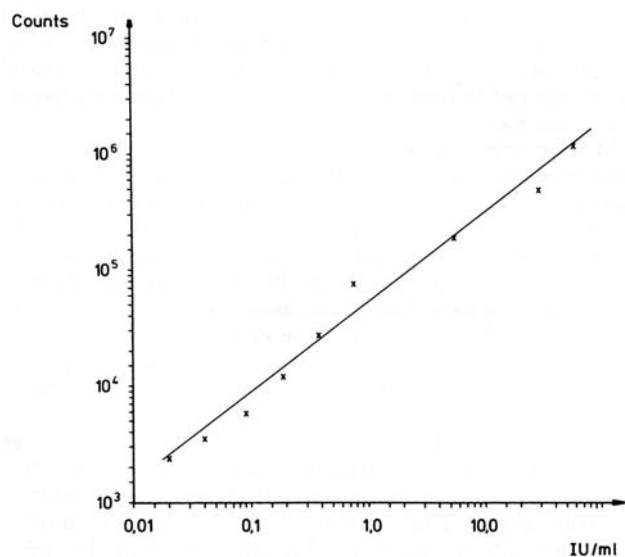


Fig. 1. Standard curve for tetanus antitoxin by using calibrated reference serum with antitoxin concentrations ranging from 0.02 to 15 IU/ml. Mean values are from duplicates; final assay dilution was 1:100.

The coefficient of variation varied between 5.8 (intra-assay) and 2.96 (inter-assay) in sera with 2.5 IU of antitoxin per ml. The intra-assay and interassay coefficients of variation were higher (15.5 and 17.0, respectively) in sera with low levels of tetanus antitoxin. Intra-assay and inter-assay variations reflecting the precision of the test are shown in Table 2.

The TR-FIA was compared with the ELISA technique by parallel testing of 90 serum specimens from patients with unknown tetanus antitoxin levels. A very good correlation coefficient was found ($r = 0.9421$), with a line of best fit of $y = 0.9127x + 0.0218$, where $y =$ ELISA and $x =$ TR-FIA. A comparison of the results of various patients measured by the ELISA and the TR-FIA is shown in Fig. 2.

TABLE 2. Comparison of the intra- and interassay variations by using sera of various tetanus antitoxin concentrations

Material	Intra-assay			Interassay		
	\bar{x}	SD	CV ^a (%)	\bar{x}	SD	CV (%)
Reference standard	2.547	0.149	5.85	2.426	0.072	2.96
High-level antitoxin	6.54	0.634	9.69	6.7	0.686	10.2
Mean-level antitoxin	1.738	0.187	10.7	1.71	0.197	11.52
Low-level antitoxin	0.122	0.019	15.5	0.123	0.021	17.0

^a CV, Coefficient of variation.

Because of uncertain protective immune status with regard to tetanus, the tetanus antitoxin level in 2,630 men between 19 and 90 years of age was investigated. Table 3 demonstrates the age-dependent distribution of tetanus antitoxin in 2,630 men. About 60% of the men between 19 and 60 years of age revealed titer levels from 1.1 to 10 IU/ml. In contrast,

only 5% had titer levels lower than 0.1 IU/ml. In the men between the ages of 60 and 90 years, an increase in those with titer levels lower than 0.1 IU/ml was found.

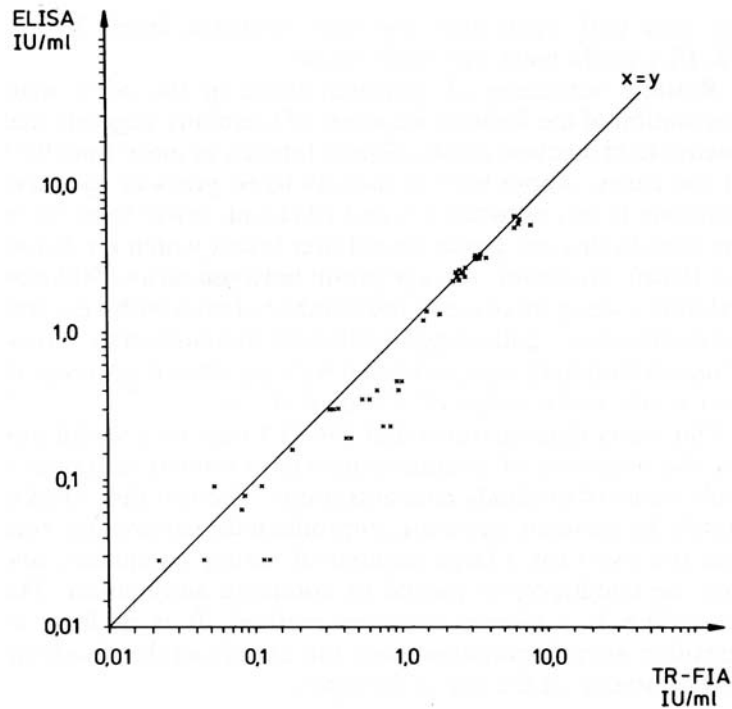


Fig. 2. Comparison of results measured in ELISA and in TR-FIA.

TABLE 3. Age distribution of defined tetanus antitoxin titers in men

Antitoxin (IU/ml)	No. of men with tetanus antitoxin titer in age group (yr)										Total no.
	19-25	26-30	31-35	36-40	41-45	46-50	51-60	61-70	71-80	81-90	
0.001-0.01	24	6	4	3	1	2	5	14	16	3	78
0.011-0.1	19	8	3	1	2	1	4	5	8	1	52
0.11-1.0	203	46	22	16	12	39	40	15	9	5	407
1.1-10	942	340	81	48	42	65	90	4	5	3	1,620
>10	352	80	16	5	10	9	1	0	0	0	473
Total no.	1,540	480	126	73	67	116	140	38	38	12	2,630

Discussion

Although vaccination with tetanus toxoid is mostly well tolerated, adverse side effects can appear in some cases, usually related to hyperergic reactions (4, 21). To avoid hyperergic reactions following tetanus toxoid revaccination, the tetanus antitoxin level should be determined before a booster application. To this aim, ELISA techniques are widely employed.

To increase both sensitivity and measurement range of tetanus antitoxin assays, we tried modifications of the standard ELISA technique, e.g., by a coupled enzyme procedure analogous to assays in clinical chemistry. Thus, a special ELISA intensification system for alkaline phosphatase (BRL, Cambridge, United Kingdom) was applied in which alkaline phosphatase generates a product which initiates a secondary cyclic enzyme reaction, leading to a colored product. Unfortunately, this procedure was accompanied by an extreme diminution of the measurement range. Because of the impracticability of handling at least duplicate approaches (different measuring ranges) and high costs, this method was not followed up.

In view of this experience, a TR-FIA for the detection of IgG antibodies to tetanus toxoid was evaluated. The amount of tetanus antitoxin was quantified by dissociating the Eu^{3+} from antibodies into the fluorescence enhancement solution. The ion forms a highly fluorescent chelate simultaneous with its liberation (9, 10). The TR-FIA finally replaced the ELISA in our routine work and is now employed as the method of choice for the serological evaluation of vaccination states.

The fundamental properties of the TR-FIA indicate a great potential for antibody detection over wide quantitation ranges (10). This range enables the measurement of extreme concentrations by use of a single dilution even in concentrations which are below the detection limit of ELISAs. With respect to quantitative data in the reliable measuring range of the conventional ELISA, good correlations were observed for both methods. TR-FIA has been found to be a practical, rapid, and economic method for estimating tetanus antitoxin in human sera, having specificity similar to that of the ELISA. The Eu^{3+} labels are nontoxic and stable (13). Moreover, the accuracy of the estimates of antitoxin content for sera with both high and low antitoxin levels by the TR-FIA could meet our expectations.

Routine screening of antitoxin titers in the adult male population of the Federal Republic of Germany suggests that adequate protection exists against tetanus in more than 95% of the cases. About 60% of men 19 to 60 years of age have antitoxin levels between 1.1 and 10 IU/ml; fewer than 5% of the men in this age group reveal titer levels which are below 0.1 IU/ml. However, the age group between 60 and 90 years exhibits a steep increase in the number of men with very low antitoxin titers, indicating insufficient immunization status. This development was paralleled by a significant decrease of titer levels in the range of 1.1 to 10 IU/ml.

Our study demonstrates that TR-FIA may be a useful tool for the detection of tetanus antitoxin in human sera over a wide range of antibody concentrations. The test described is simple to perform, accurate, reproducible, suitable for routine use even for a large number of serum specimens, and may be conducive to partial or complete automation. The procedure is a simple two-step method. It is at least as sensitive as radioimmunoassays but avoids all the disadvantages entailed in the use of isotopes.

Acknowledgments: We gratefully acknowledge the helpful suggestions of U. Gross and the critical reading of the manuscript by Jürgen Heesemann.

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