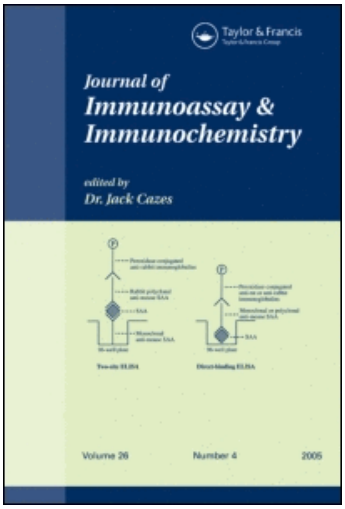

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Comparison of ELISA and Toxin Neutralization for the Determination of Tetanus Antibodies

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COMPARISON OF ELISA AND TOXIN NEUTRALIZATION FOR THE DETERMINATION OF TETANUS ANTIBODIES

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ABSTRACT

In a sandwich ELISA for tetanus antibodies, the influence of the tetanus toxoid concentration used for coating microtiter plates has been studied. The antibody levels by toxin neutralization bioassay and by ELISA were studied for a population with known immunization history. By decreasing the tetanus toxoid concentration in ELISA from 5 to 0.2 Lf/ml, a better correlation was found between the ELISA results and the bioassay titers, but sera from recently immunized people still showed high ELISA titers. This phenomenon cannot be ascribed to nonspecific reactions since sera from non-immunized people are negative in both assays. All sera negative in ELISA had, however, a bioassay titer beneath 0.01 IU/ml.

Key words: antibodies to tetanus-ELISA versus toxin neutralization-optimalization-avidity

INTRODUCTION

Traditionally, a bioassay method is used for the determination of tetanus antitoxin. To avoid the use of experimental animals, we tried to develop an in vitro test to determine the level of antitoxins in human sera. Various assays have already been described (1), indirect hemagglutination (2, 3), latex agglutination (4), and agar gel precipitation (5, 6). However, these tests lack sufficient sensitivity or have poor reproducibility. Radioimmunoassay (7) and

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ELISA (8) have also been investigated. Both assays are sensitive, reproducible, and easy to perform. A number of investigators studied the relation between RIA or ELISA and other *in vitro* tests (9, 10, 11). Only one study deals with the relation between ELISA and the toxin neutralization bioassay (12).

We describe here investigation of different types of human sera by ELISA. Good agreement between ELISA and toxin neutralization was found with hyperimmune sera, but the correlation was poor with sera taken from people shortly after immunization. Lehtonen and Viljanen (13) showed that the concentration of antigen used affects the sensitivity of ELISA for antibodies of different avidities. For that reason we tried to improve the results of the ELISA by modifying the toxoid concentration in the first step of the assay.

MATERIALS

For ELISA, microtiter plates of special quality were used: polyvinylchloride plates, type III (Flow laboratories, Scotland).
Tetanus toxoid was a purified product as routinely used for vaccine production (purity 1850 Lf/mg protein nitrogen).
Bovine serum albumin (BSA) was obtained from Povite (Oss, The Netherlands), Tween 80 from Merck (München, West Germany).
Anti human immunoglobulin was prepared by hyperimmunization of sheep with human IgG, emulsified in Freund's complete adjuvant. It contained antibodies to light as well as to heavy chains. Specific anti human IgG heavy chain serum was made from it by absorption

with $F(ab')_2$-fragments, made by peptic digestion of human IgG (14). The sera from 22 healthy adults before, during and after their immunization with tetanus vaccine were tested by ELISA at the following times: (i) pre-immunization, (ii) day 28 (4 weeks after first immunization), (iii) day 49 (3 weeks after second immunization) and (iv) day 210 (6 months after second immunization).

A human serum pool having 10 IU/ml (neutralizing antibodies) was used as reference serum in the ELISA. This serum was diluted with normal human serum without detectable antitoxin to give concentrations of 10, 8, 6, 4, 2, 1, and 0,4 IU/ml.

METHODS

a. Bioassay of tetanus antitoxin was performed as described by Ipsen (15), on a titration level of L+/10.000.

b. Anti-human IgG heavy chain antiserum was purified by the caprylic acid precipitation method of Steinbuch and Audran (16), and conjugated with horse radish peroxidase (Sigma, type VI) accordin to Nakane and Kawaoi (17), omitting the final sodium borohydride reduction (18). It was freeze-dried in the presence of 1% BSA and 2% lactose and stored in vacuum sealed ampoules.

c. Performance of ELISA: Microtiter plates were incubated for 16 hours at room temperature with 0.1 ml of different toxoid dilutions in sodium chloride, 0,9 g/L, in each cup. The plates were washed with Tween 80 in tap water (0.3 ml/L), and 0.1 ml of sera or reference solutions, all diluted 1:300 in BSA 5 g/L in saline 9 g/L with Tween 80, 0.1 ml/L (saline diluent), were added and incubated for 2 hours at 37°C. The plates were washed

and 0.1 ml antihuman IgG-peroxidase conjugate diluted 1:500 in saline diluent was added to each well and incubated for 2 hours at 37°C. The plates were washed again and the peroxidase activity bound was determined by addition of 0.1 ml 5-amino-salycilic acid (800 mg/L) with H_2O_2 (0.5 ml/L). The reaction was stopped after 10 minutes by addition of 0.1 ml of 0.33 N NaOH. The optical density was measured at 450 nm after 10 minutes in a vertical beam spectrophotometer (Multiscan, Flow labs, Scotland). The results obtained with the dilutions of the reference were plotted against the antibody concentrations. Unknown samples were read from this calibration curve and results expressed in antitoxic units (AU) per ml. All assays were done in duplicate. To study the influence of the toxoid concentration on the ELISA results the microtiter plates were incubated with tetanus toxoid at concentrations of 20, 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04, 0.02 and 0 Lf/ml. After 18 hours incubation at room temperature, the tetanus toxoid concentration remaining in the supernatant and in the first two washing fluids was determined with a toxoid ELISA. Sheep IgG anti tetanus toxoid was used to coat the wells and also for the peroxidase conjugate. Incubations and wash procedures were similar to those described for the antibody determination.

RESULTS

The amount of tetanus toxoid adsorbed to the pvc plate was calculated by subtracting the tetanus toxoid concentration in the supernatant and in the two washing fluids from the original toxoid

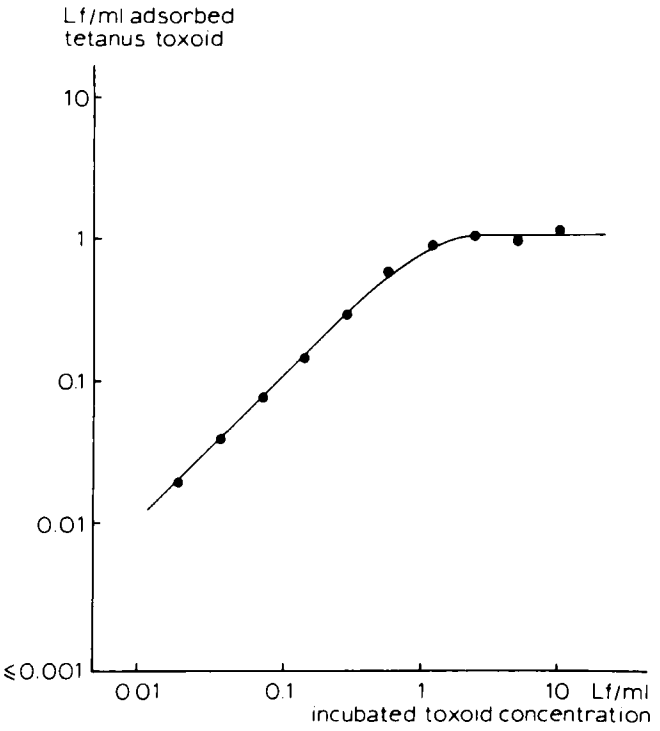

FIGURE 1. Adsorption of tetanus toxoid to the solid phase.

concentration (figure 1). Adsorption is nearly complete at concentrations up to 1.0 Lf/ml and reaches saturation at 2 Lf/ml.

The plates from this experiment were used to study the behaviour of sera showing descrepant results between ELISA and bioassay:

serum 1: a serum of a hyperimmunized individual, with a high bioassay titer (30 IU/ml) and a high ELISA titer (20 AU/ml).

serum 2: a serum with a high ELISA titer (27 AU/ml) and a low bioassay titer (0.023 IU/ml).

serum 3: a serum without detectable antitoxins in either assay.

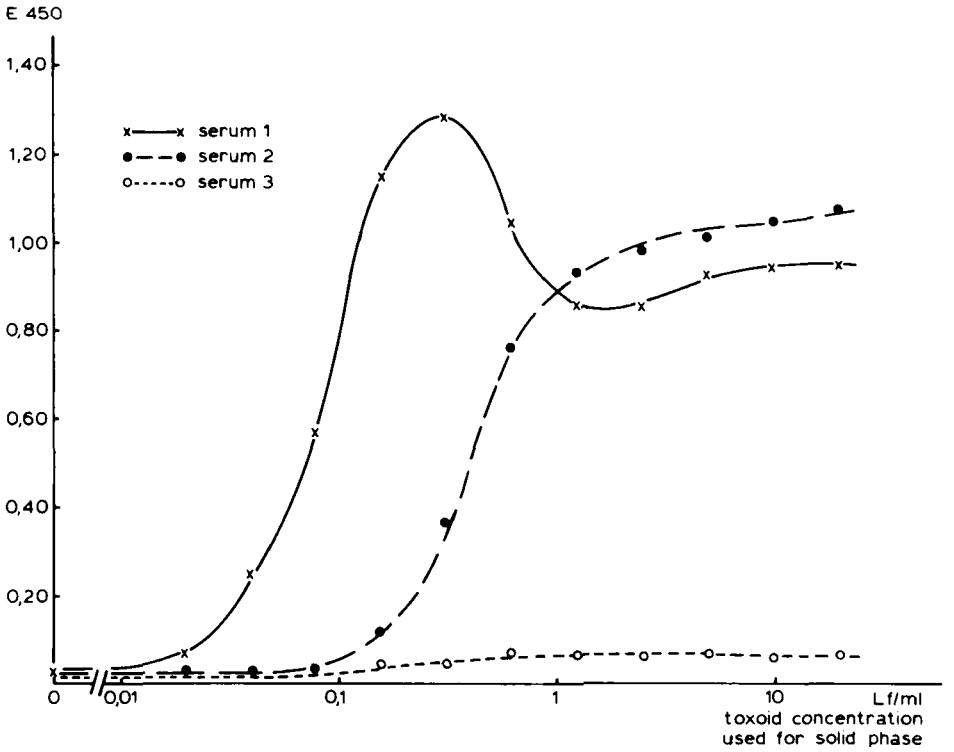

FIGURE 2. Absorbance units obtained in ELISA for 3 different sera: diluted 300 times; conjugate diluted 500 times
Serum 1: serum of a hyperimmune person
Serum 2: serum with a very high ELISA titer and a low bioassay titer
Serum 3: negative serum

The results obtained with these sera are shown in figure 2. The toxoid concentration used for coating the plates has a strong influence on the optical density measured. Using high toxoid concentrations there was only a small difference between the ELISA absorbances of serum 1 and 2, whereas at low toxoid concentrations the difference was large. The negative serum had a low optical density at all toxoid concentrations tested.

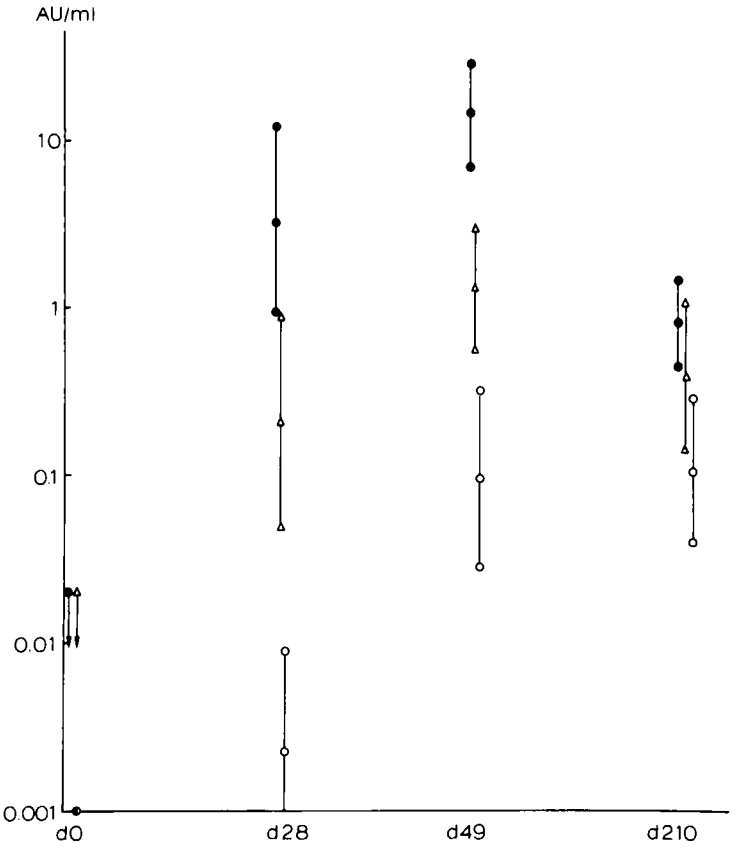

FIGURE 3. Comparison of antibody results by bioassay (O) (detection limit (0.001 AU/ml), low toxoid ELISA (Δ) and high toxoid ELISA (●) (both detection limits 0.01 AU/ml) in 22 subjects at 0, 28, 49 and 210 days after immunization. All results shown as GMT ± 1 s.d.

Based on the results of these experiments we selected two toxoid concentrations for coating the plates: 5 Lf/ml and 0.2 Lf/ml, the first giving high absorbances for both sera, the second giving high values only with the hyperimmune serum.

The sera from the immunization study were assayed, using these two toxoid concentration. The results are presented in figure 3.

Using the high toxoid concentration, ELISA results exceeded those of bioassay by factors of 2000, 150 and 6 at 28, 49 and 210 days post immunization respectively. With the low toxoid concentration, the corresponding factors were 150, 11 and 3.

DISCUSSION

ELISA is a sensitive and simple assay which allows titration of many samples in one day. Unlike the toxin neutralization bioassay, it does not require experimental animals. There are however, significant differences between the results obtained by these two assays. No serious problems will be encountered when hyperimmune sera are assayed but sera from recently immunized people will give higher results with ELISA (19). Without knowing the origin of the sera, it will be difficult to estimate the toxin neutralization activity from ELISA data.

In our studies a peak in the optical density curve was found for an hyper-immune serum at a non-saturated coat (0.3 Lf/ml) instead of a continuous decrease as might be expected (fig. 2), and other hyper-immune sera showed similar effects. This may be due to concentration dependent sterical arrangements of the toxoid molecules on the solid phase. The maximal distance between the absorbance curves of the hyperimmune and low bioassay titre sera was found at a toxoid concentration of 0.2 Lf/ml, and this concentration of toxoid was chosen for coating the microplates to improve the correlation between ELISA and bioassay results. Even at this concentration, sera from recently immunized people still

had a higher antibody result by ELISA than by bioassay. Such sera may have low avidity antibodies, more reactive in ELISA than in bioassay. Any further decrease in toxoid concentration results in unacceptably low assay absorbances.

Since avidity normally increases with repeated vaccination, ELISA may be used in longitudinal studies in which various samples from one individual are to be compared. Also the ELISA will give useful information in epidemiological studies in which the effect of immunization in a population is to be studied. Additional studies are needed to develop an ELISA system equivalent to the toxin neutralization bioassay in mice for sera taken shortly after vaccination. Sera negative by ELISA are always negative by bioassay, and probably originate from people who have never been vaccinated with tetanus vaccine. This ELISA can demonstrate antibody production at an early stage, before neutralizing antibodies can be detected.

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