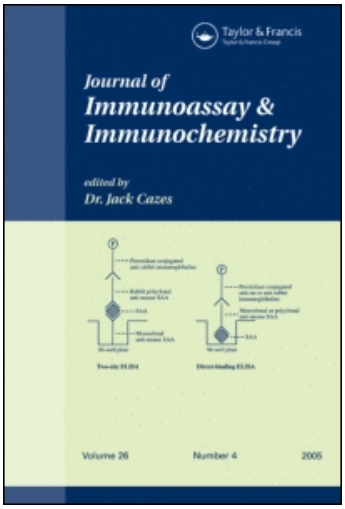

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 09 February 2004

To cite this Article Šeatović, Saša , Inić-Kanada, Aleksandra , Stojanović, Marijana , Živković, Irena , Jankov, Ratko M. and Dimitrijević, Ljiljana(2005) 'Development of Sandwich Enzyme-Linked Immunosorbent Assay for Determination of Tetanus Toxoid Concentration', Journal of Immunoassay and Immunochemistry, 25: 1, 31 — 44
To link to this Article: DOI: 10.1081/IAS-120027224
URL: http://dx.doi.org/10.1081/IAS-120027224

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 25, No. 1, pp. 31–44, 2004

Development of Sandwich Enzyme-Linked Immunosorbent Assay for Determination of Tetanus Toxoid Concentration

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ABSTRACT

According to the recommendation of the World Health Organization (WHO), the use of an in vivo test for measuring of the potency of tetanus toxoid vaccine (TTdV) is still unavoidable, but the establishment of a convenient in vitro test would significantly improve the work in this field. A sandwich enzyme-linked immunosorbent assay (sELISA) was developed for a rapid and sensitive quantification of tetanus toxoid (TTd).

We produced four monoclonal antibodies (MAbs) designated 41, 51, 62, and 71 that reacted with TTd and recognized different antigenic determinants on TTd. We also used two of these antibodies for developing a sELISA, with MoAb 71 as an immobilized and MoAb 51 as a

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DOI: 10.1081/IAS-120027224 1532-1819 (Print); 1532-4230 (Online)

capture antibody. The measurement range of this assay was from 31–1000 ng/mL and the minimum detection limit for TTd was 31 ng/mL. This high sensitivity of this sELISA and its good reproducibility suggest that the developed method could be reliably used to estimate the concentration of TTd, which could be easily extrapolated to the estimation of vaccine potency.

Key Words: Sandwich ELISA; Tetanus toxoid; Monoclonal antibodies.

INTRODUCTION

Tetanus toxin (TTn) is an extremely potent neurotoxin[1] produced by the anaerobic bacterium, *Clostridium tetani*. The active holotoxin of tetanus (150 kDa) is comprised of two polypeptide chains, termed heavy (H, 100 kDa) and light (L, 50 kDa) chains, linked by a disulphide bond. Tetanus toxin traffics to the central nervous system by retrograde axonal transport, followed by trans-synaptic spread into inhibitory inter-neurons[2,3] and, at a concentration of 2.5 ng/kg, induces death.

As TTn induces death before adoptive immunity could be generated, active immunization[4] with tetanus vaccine is inevitable for prevention of death caused by tetanus. Nowadays, protection is routinely induced by immunization with TTn derivative, tetanus toxoid (TTd), obtained by chemical modification.[5] However, cross-reactivity of TTn with some human proteins, such as β_2-glycoprotein I, which has been shown as one of the major antigens in phospholipid syndrome,[6] raises a possibility of induction of some "unexpected" and potentially dangerous antibodies. This fact shows that application of TTd needs prior detailed characterization and precise quantification.

According to the recommendation of the WHO, the use of an in vivo test for measuring of the potency of TTd vaccine (TTdV) is still unavoidable, but the establishment of a convenient in vitro test would significantly improve the work in this field. Immune assessments of TTdV are based on the measurement of antitoxin immunity and, traditionally, the most widely[7] accepted methods have been functional models assessing the neutralizing activity of antitoxin only. For the titration of tetanus antitoxin, the toxin-neutralization test in mice or guinea-pigs has proved to be a reliable model.[8] For ethical, economical, and practical reasons, there is growing interest for in vitro models for determination of TTd concentration in TTdV which could be extrapolated to the vaccine potency. A prerequisite is that such models are reproducible, and can detect the lowest levels of toxoid which can provide protection.

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This work was undertaken with the aim of developing an in vitro test for the determination of TTd concentration in TTdV. Herein, we present a sandwich enzyme-linked immunosorbent assay (sELISA) which offers advantages of speed, specificity, and simplicity over some other in vitro tests, e.g., toxin-binding inhibition test[7] or indirect ELISA, and can measure protein concentration in TTdV down to 30 ng/mL.

EXPERIMENTAL

Antigens

Production of TTd, which is the active component of TTdV, includes fermentation, detoxification using formaldehyde, and ammonium sulphate precipitation followed by gel-exclusion chromatography on a Superose 12 column (FPLC System, Pharmacia, Uppsala, Sweden). TTd produced at our Institute was assigned as TTdT. The International Standard for Tetanus Toxoid was obtained from StatenSerum Copenhagen (TTdS).

Production of Monoclonal Antibodies Specific for Tetanus Toxoid

In order to obtain TTd-specific monoclonal antibodies (MAbs)-secreting clones, we immunized 6-week-old (female) BALB/c mice with TTdT [immunization was performed with 100 μg of TTdT/dose administered three times at one-week intervals, emulsified in complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and phosphate buffer saline (PBS), respectively]. Three days after the final immunization, spleen cells (2×10^8) from each mouse were prepared and fused to a mouse Sp2/mIL-6 cell line in the presence of polyethylene glycol 1000 (Gibco BRL), 1 g/mL. After 1 min, the cell suspension was diluted with serum-free RPMI 1640 medium (Gibco BRL) and the cells were collected via centrifugation at $1100 \times g$ (Hettich Centrifuges, Tuttingen, Germany) for 5 min. The cell pellet was suspended in a RPMI 1640 medium containing 20% fetal calf serum (FCS) (HyClone) and seeded into a 96-well microtiter plate. After 24 hours, 100 μL/well of medium containing HAT (Sigma) was added. Plates were incubated in a 5% CO_2 atmosphere at 37°C (Heraeus, Hanau, Germany) for 2 weeks. Hybridomas producing anti-tetanus MAbs were selected by testing the hybridoma culture medium with an ELISA that used TTdT and TTdS fixed to a 96-well microtiter plate (Maxisorb plates, Nunc). A solution of 1% BSA (Sigma)/PBS was used for saturation (200 μL/well, 1 h at room

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temperature), preparation of samples and dilution of biotin conjugated (-B) anti-mouse IgG (Sigma), and streptavidin-peroxidase (Sigma). Following saturation, the plates were washed with 0.05% Tween 20 (Sigma)/PBS ($4 \times 200\,\mu L$/well) and PBS ($2 \times 200\,\mu L$/well). Supernatants were incubated at room temperature for 1 h. Plates were washed, and 50 μL/well of anti-mouse IgG-B was added and incubated 1 h at room temperature. After washing, 50 μL/well streptavidin-peroxidase diluted 1 : 1000 was added for 1 h at room temperature and washed again. The wells were finally incubated with 50 μL/well substrate solution containing *o*-phenylendiamine (OPD) (Sigma) and H_2O_2 (Merck) at room temperature for 5 min. The reaction was stopped by addition of 50 μL/well of 2 M H_2SO_4 and absorbance was measured at 492/620 nm (Multiscan Ascent, Labsystems, Helsinki, Finland) microplate reader. All other ELISA tests applied in this work used the same saturation, washing, enzyme dependent color development, and stopping conditions.

Ascites fluid containing concentrated MAbs was produced by interperitoneally injecting hybridomas into BALB/c mice treated with pristane (Sigma). IgGs were purified from ascites fluid by ammonium sulphate fractionation and protein A (Pharmacia, Uppsala, Sweden) affinity chromatography. IgG (all MAbs established belonged to IgG1 class which was determined by ELISA using biotin labeled antibodies specific for murine IgG subclasses, purchased from Sigma) was eluted from the column (2 mL) with 0.1 M citric acid (Merck), pH 6.0. The pH of the eluate was adjusted to pH 7.0 and dialyzed against PBS and 0.1 M $NaHCO_3$ (ZorkaPharma, Sabac, SCG). The purity of MAb preparations was determined by SDS-PAGE (Phast System, Pharmacia LKB, Uppsala, Sweden).

Immunoblotting

For immunoblotting, TTdT and TTdS were resolved by SDS-PAGE on 1-mm-thick 9% separating gels, with 4% stacking gels (Mini Protean II System, Bio-Rad, USA). Proteins were electrophoretically transferred to PVDF (Millipore Corporation, Bedford, MA, USA) for 1 h at 4°C and $1-1{,}5\,mA/cm^2$ (Multiphor II System, LKB, Sweden) in buffer containing 25 mM Tris (Pharmacia), 193 mM glycine (Pharmacia), and 15% methanol (Fluka). To prevent nonspecific antibody binding, membranes were incubated with blocking buffer, 3% BSA (Sigma) in PBS for 2 h. Antibodies were diluted in blocking buffer and incubations were for 1 h at room temperature. Membranes were incubated with anti-mouse IgG-B (Sigma) diluted 1 : 5000, followed by an alkaline phosphatase (Sigma) diluted 1 : 1000. Membranes were washed three times with 0.05% Tween 20 (Sigma)/PBS and twice with

PBS after each incubation. Antibody binding was visualized by exposure to 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Bio-Rad).

Determination of Additivity Index

MAbs were diluted particularly to 2.5 μg/mL and added separately and together at saturating concentration (determined by an indirect ELISA procedure) to a 96-well microtiter plate (Maxisorb plates, Nunc) coated with TTdT at a concentration of 1 μg/mL (50 μL/well, at 4°C overnight). After saturation and washing, anti-mouse MAb-B (Sigma) was added and incubated for 1h at room temperature. Streptavidin-peroxidase (Sigma) complex formed after one hour incubation was detected in the presence of OPD (Sigma) and measured at 492/620 nm (Multiscan Ascent, Labsystems, Helsinki, Finland) microplate reader.

Sandwich Enyzme-Linked Immunosorbent Assay for Determination of Tetanus Toxoid Concentration

The ELISA was based on a sandwich method linking two MAbs against TTd assigned as 51 and 71. A 96-well microtiter plate (Maxisorb plates, Nunc) was coated with MAb 71 in concentration of 2.5 μg/mL (50 μl/well, at 4°C, overnight). After saturation and washings, the wells were incubated with serial dilutions of TTdT or TTdS (31–1000 ng/mL) and diluted samples of vaccines, 50 μL/well at room temperature for 1 h. The complex formed between MAb 71 and TTdT or TTdS or diluted samples of vaccines was detected in the presence of MAb 51-B at a final concentration of 2.5 μg/mL, 50 μL/well, after 1 h incubation at room temperature followed by streptavidin-peroxidase (Sigma) and OPD (Sigma). All assays were performed in duplicate.

The concentration of TTd in vaccine sample was determined by comparing the sample absorbance with absorbance obtained from the calibration curve of TTdT used as a standard. TTdT was used instead of TTdS for the following reasons: preliminary tests which were performed with both toxoids simultaneously showed identical dependance of 492/620 nm absorbance on TTd concentration (data not shown). On the other hand, the source of TTdT was unlimited as it was produced at our Institute.

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Statistical Analysis

To determine the intra-assay precision, 12 replicates of the same sample were analyzed in a single analytical run. The inter-assay precision was determined by analyzing aliquots from a single sample on 6 different days over a 1-month period. The level of variation of TTd concentration for each tested vaccine was determined by calculating the coefficient of variation (CV), defined as the respective standard deviation (SD) divided by the overall mean (MV) and expressed as a percentage.

RESULTS

Monoclonal Anti-tetanus Toxoid Antibodies Characterization

Four antibodies, assigned as 41, 51, 62, and 71, displayed strong reactivity against TTdT and TTdS. Western blot analysis revealed that these antibodies recognize the TTd band of 150 kDa and lower molecular mass bands of approximately 100 and 50 kDa (Fig. 1).

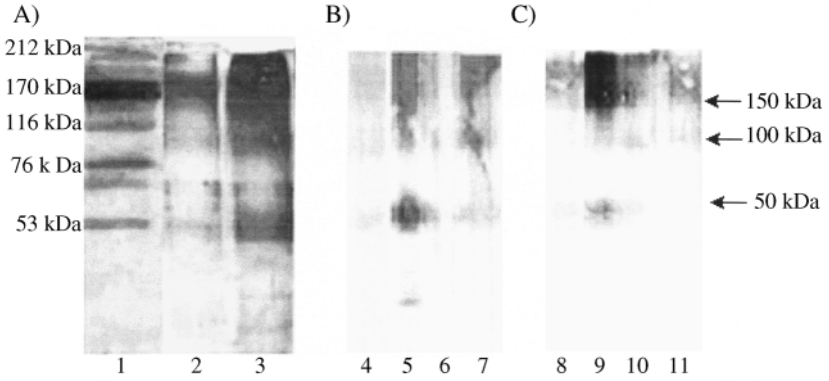

Figure 1. (A) Molecular weight standards (line 1), TTdT (line 2), TTdS (line 3) were subjected to SDS-PAGE on 9% polyacrylamide gel. (B) Western blot analysis of reactivity of MAb secreting clones 41 (lines 4 and 8), 51 (lines 5 and 9), 62 (lines 6 and 10), and 71 (line 7 and 11) toward TTdT and TTdS, respectively. The molecular weight marker kit consists of myosin (212 kDa), α-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa).

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Optimization of the Sandwich Enyzme-Linked Immunosorbent Assay

To test the ability of two MAbs to bind simultaneously on the different epitopes of the same antigen, we first determined their additivity indices. The values of the additivity indices obtained with 4 MAbs generated against TTdT are given in Table 1. High additivity indices (>40) indicate pairs of MAbs that can bind simultaneously to the TTdT or TTdS, while low additivity indices (<20) indicate those pairs that cannot. Based on the MAbs additivity indices, we selected the following antibody pairs of MAbs which could be used for sELISA: 41 + 51, 51 + 62, and 51 + 71. The other pairs were not applicable for the following reasons: their low additivity indices or their limited optical density level. The additivity test defined MAb 51 and MAb 71 as the optimal pair of antibodies for sELISA for the determination of TTd concentration with an additivity index 82 for TtdS and 88 for TTdT, respectively. The specificity of anti-TTdT MAbs 51 and 71 was further ascertained by immunoblotting (Fig. 2). These two MAbs reacted with TTdT revealed as a band of 150 kDa, but their binding specificity for lower molecular mass proteins which exist in TTdT or TTdS as a consequence of detoxification differ. Western blot with MAb 51 and MAb 71 also showed additional bands which contain a molecular mass of 50 kDa and 100 kDa, respectively. This finding can indicate that epitopes for MAb 51 and 71 are located on different chains of TTd.

In order to further establish coated versus capture antibody for the use in sELISA, we tested different pairs of MAbs specific for TtdT. The six tests were done [coated Ab-Ag-capture Ab]: MAb51-TTdT-MAb41-B; MAb51-TTdT-MAb62-B; MAb51-TTdT-MAb71-B; MAb41-TTdT-bMAb51-B; MAb62-TTdT-MAb51-B; MAb71-TTdT-MAb51-B and, after we performed analyses, the following results were obtained (Fig. 3).

Table 1. Additivity indices for MAbs 41, 51, 62, and 71 specific for TTdS and TTdT.

	AI	
MAb	TTdS	TTdT
41 + 51	72	68
41 + 62	15	92
41 + 71	37	86
51 + 62	53	67
51 + 71	82	88
62 + 71	11	35

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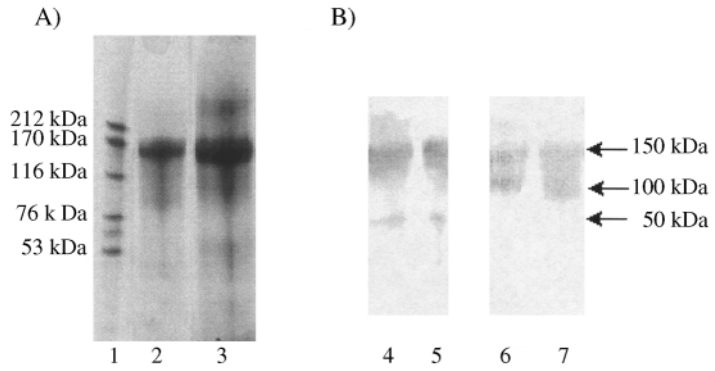

Figure 2. SDS-PAGE (A) and immunoblotting (B) with biotin conjugated MAb 51 and 71. (A) TTdT and TTdS were resolved on a 7.5% polyacrylamide gel. The first, the second and the third line correspond to molecular weight standards, TTdT and TTdS, respectively. Each gel was fixed and CBB stained. (B) TTdT and TTdS were transferred onto PVDF and immunoblotted with MAbs 51 and 71. Lines 4 and 5 show the specificity of MAb 51 for TTdT and TTdS, respectively. Lines 5 and 6 show the specificity of MAb 51 for TTdT and TTdS, respectively.

Based on the above data, we selected MAb 71 as a capture antibody. sELISA with MAb 41 as a coated antibody was abandoned as it showed limited optical density and the sELISA with MAb 62 was excluded as MAb 62 showed unusual sticky properties.

According to all we have mentioned above, we selected MAb 71 and MAb 51-B as an antibody pair combination for the determination of TTd concentration in our sELISA.

Enzyme-Linked Immunosorbent Assay Performance

For calibration of the sELISA test for the determination of TTd concentration, the standard was TTdT, with tetanus concentration expressed in μg/mL, determined by Lowry. The calibration curve, constructed from a set of six dilutions of the TTdT, was used in our laboratory to determine the TTdT concentration in investigated vaccines. A calibration curve (Fig. 4) for each assay was constructed with sixth dilutions of TTdT in concentrations ranging from 0.031 to 1 μg/mL and TTdT concentration in the investigated vaccine was calculated with data analysis software. There was a good linear relationship between the dilution ratio and concentration of TTd in TTdV detected by sELISA with the minimum detection limit for TTdT of 31 ng/mL.

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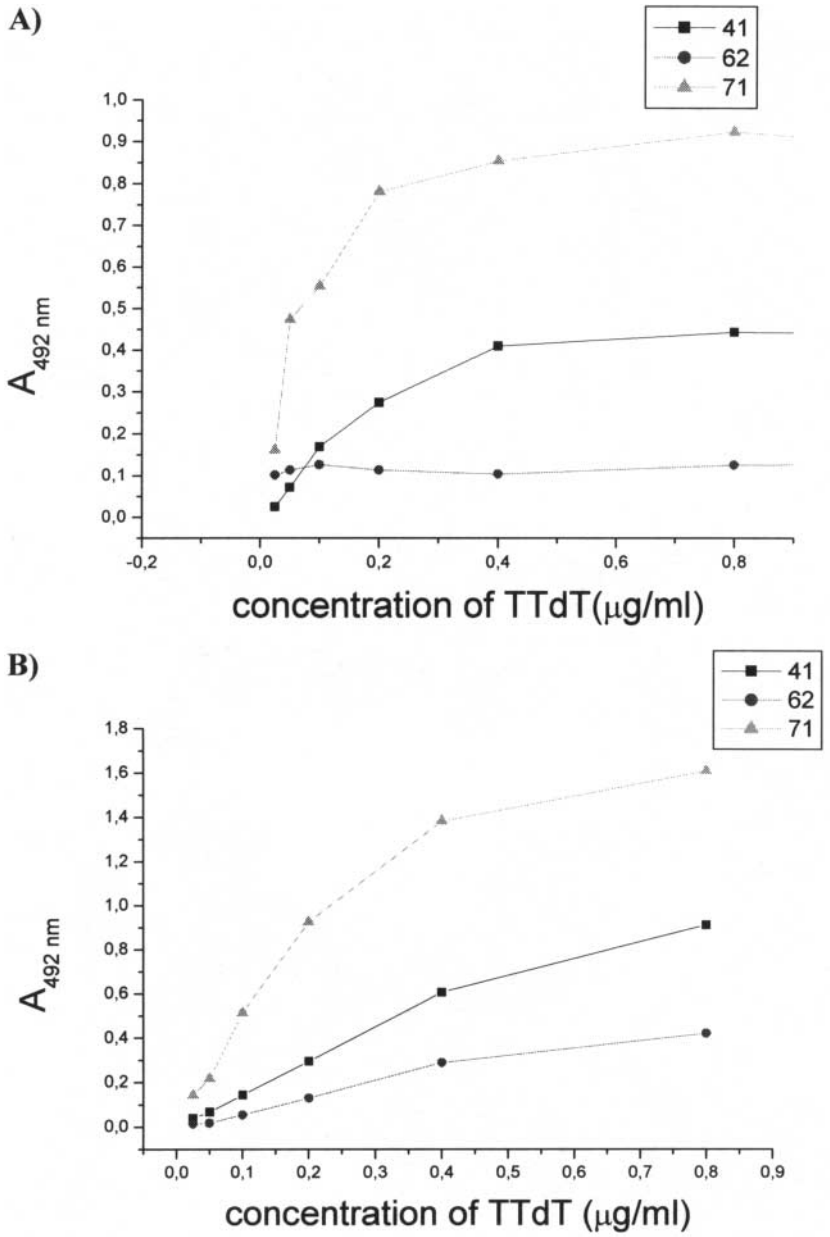

Figure 3. The sELISA for establishing an optimal combination of antibodies for the determination of TTd concentration: (A) MAb 51 was used as a coated antibody and MAb 41-B, MAb 62-B, MAb 71-B as capture antibodies; (B) MAb 41, MAb 62 and MAb 71 were used as coated antibodies and MoAb 51-B as a capture antibody. Assay were done as described in Material and Methods.

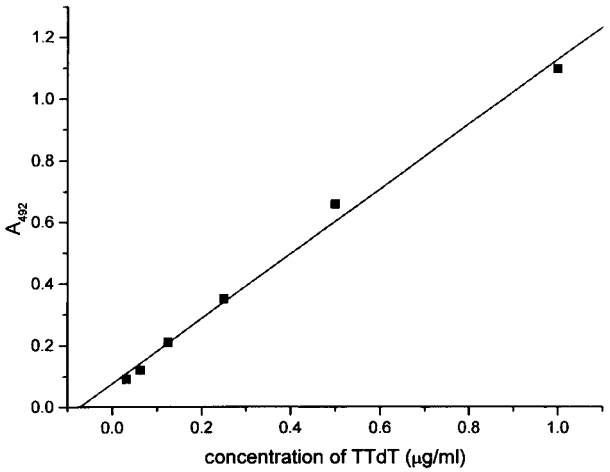

Figure 4. The standard curve for sELISA developed for the determination of TTd concentration.

During the development of the sELISA, we used three commercial vaccines. The average value for each vaccine was estimated after assay.

In the intra-assay ($n = 12$), the means of three vaccine samples were 4.29, 3.85, and 4.83 μg/mL and their CVs were 6.76%, 3.89%, and 2.48%, respectively. In the inter-assay ($n = 12$), the means of three vaccine samples were 3.92, 4.18, and 5.28 and their CVs were 7.65%, 5.26%, and 5.11%, respectively (Table 2).

Table 2. sELISA precision test for the determination of TTdT concentration.

Sample	Mean (μg/mL)	SD (μg/mL)	CV (%)
Intra-assay validation			
Vaccine 1	4.29	0.29	6.76
Vaccine 2	3.85	0.15	3.89
Vaccine 3	4.83	0.12	2.48
Inter-assay validation			
Vaccine 1	3.92	0.30	7.65
Vaccine 2	4.18	0.22	5.26
Vaccine 3	5.28	0.27	5.11

No signals were recorded, either with diphtheria toxoid or pertussis toxin as antigens, which indicated that our sELISA could be used for determination not only of TTd concentration in Te-Al vaccine but TTd concentration in multicomponent vaccine (e.g., DiTePer) as well.

DISCUSSION

The main purpose of the present study was to develop an ELISA for measuring of TTd concentration in TTdV. Once the test for the determination of TTd concentration is established it could be easily extrapolated to the measurement of vaccine potency and, thus, provides an alternative to in vivo tests.

Problems with the in vivo tests on animal models are well known: price, long duration, influencing factors, and extrapolation to the human system. On the other hand, in vitro tests require specific reagents and detailed investigations of conditions necessary for obtaining valid results.

In order to obtain specific tools for qualitative and quantitative analyses of TTd, we produced several clones which secret MAb specific for TTd. We analyzed the reactivity of its supernatants toward different tetanus antigenic determinants (TAD) and obtained results implied on differences in specificity and affinity of interactions.

Our accomplishment in producing the two murine MAbs shows that we have developed the procedure for designing and producing MAbs that react with multiple epitopes of TTd, which has been demonstrated by Western blot and indirect ELISA.

As could be seen from the results of sELISA, supernatants of selected clones reacted differently with TAD. This fact could be used for discrimination between TTn and toxoid forms and for analyses of efficient transformation of toxin to non-toxic toxoid forms. Observed differences in reactivity of obtained MAbs toward toxoid forms might be the result of different extents of chemical modifications, glicosylation, and presence of low molecular weight peptides produced by proteolitic action of the TTd itself.

According to its affinity to TTd, MAbs 51 and 71 might be used for ELISA and Western blot. Results of additivity test which were done in order to check possibility of simultaneous binding to different antigenic determinants on TTd (as it was necessary for their application in sELISA), implied that they could interact with TADs without any steric disturbance, owing to recognition of epitopes at "long distance" (aditivity index >40).[9] The high value of the AI for MAb 51 and 71 indicated that these MABs could be used in sELISA, which is more specific and more sensitive than other types of ELISAs.

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The initial hypothesis[5] in developing of sELISA for measuring the content of TTd in vaccine was the fact that the neutralizing epitopes in TTd were immunodominant and that low levels of non-neutralizing antibodies were produced.

For measurements of the TTd concentration in TTdV, highly specific MAbs provide a highly sensitive and specific analytical system. We successfully utilized a sELISA, which uses a solid phase MAb 71 that reacts with the heavy chain of TTd and a capture MAb 51 that reacts with TAD located in a light chain of TTd.

The measurement range of this assay is from 31–1000 ng/mL, with the minimum detection limit for TTdT of 31 ng/mL. The concentration range of this sELISA has satisfactory characteristics for extrapolation of concentration to international units, as it has recently been shown that one IU equals to 667 ng of TTd.[10] High sensitivity of this sELISA and its good reproducibility suggest that the developed method could be reliably used to estimate the concentration of TTd, which could be easily extrapolated to the estimation of vaccine potency. As TTd is a denaturated form of TTn, and no declared control of chemical denaturation exists, caution is necessary in evaluating different preparations of vaccine. Besides, this assay represents a powerful tool for process control during vaccine preparation. Both antibodies used in this assay do not react with β_2 GP-I (data not shown) and the measurements of concentrations based on these antibodies do not take into account the possible autoantigenic determinants.

The validity of the present sELISA was verified with precision inter- and intra-assay tests. Using the procedures described in this work, we obtained inter- and intra-assay coefficients of variation of less than 7.65% for quantification of TTd.

The described sELISA, which is, indeed, a microtechnique requiring very small amounts of Ag, is easy to perform, it is less time-consuming, it costs less, does not need special equipment, and it does not require handling of animals. Many samples may be titred simultaneously and, as the health authorities and animal welfare groups are committed to reducing the numbers of animals needed in the development and testing of biological products, we propose this sELISA for the determination of TTd concentration in TTdV.

ABBREVIATIONS

TTd	tetanus toxoid
TTdV	tetanus toxoid vaccine
sELISA	sandwich enyzme-linked immunosorbent assay
TTn	tetanus toxin

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MAb	monoclonal antibody
TAD	tetanus antigenic determinants
AI	additivity index
IU	international unit

ACKNOWLEDGMENTS

We thank Slobodan Živković for excellent technical assistance. This investigation was supported by a grant 1239 from the Ministry of Science and Technology of the Republic of Serbia.

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Received June 20, 2003
Accepted September 24, 2003
Manuscript 3106

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