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A solid-phase enzyme immunoassay for the detection of tetanus toxin using human and murine monoclonal antibodies

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Three human and three murine monoclonal antibodies were tested for their reactivity to tetanus toxin and toxoid and used to establish an enzyme immunoassay specific for tetanus toxin. The dissociation constants of the monoclonal antibodies were between 3.91×10^{-9} and 8.48×10^{-12}. Two human monoclonal antibodies recognized conformation determinants on the toxin, whereas the others reacted to the heavy chain. Only a combination of antibodies of the two species allowed the development of an enzyme immunoassay for the detection of tetanus toxin with a lower detection limit of 1.2 µg/l.

Tetanus toxin has an affinity to the gangliosides of different nerve cells (WELLHÖNER *et al.* 1987). Half saturation on the gangliosides containing two N-acetylneuraminic acids is already achieved in the nanomol range, since an extremely low amount of toxin is sufficient to produce a paralyzing or lethal effect in the human or animal organism (HELTING *et al.* 1977a, b, WILDFÜHR 1959). Therefore, immunization was introduced very early to prevent this disease (BERGEY *et al.* 1933). Although the toxin is highly immunogenic, it is not suitable for immunization. Therefore detoxification was achieved only by heating and formalin treatment (KRAUS 1924). Since detoxification is never total, each batch of vaccine has to be examined for possible toxin content.

Another field of application of toxin detection is the control of toxin-producing bacteria cultures in vaccine production (OZUTSUMI 1985). The most common method is toxin detection using mice or guinea pigs for subcutaneous or intramuscular injection of a defined sterile amount of the native or detoxified solution mixed with a defined amount of standardized tetanus antitoxin. The midlethal toxin dose is about 4 ng/kg mice (GORETZKI *et al.* 1985).

The defined antitoxins permit semiquantitative determination of toxin (WILDFÜHR 1959, OZUTSUMI *et al.* 1985). In addition, the toxoid is quantified and assessed by precipitation unit determination (WILDFÜHR 1959), by the KRAUS inhibition experiment or by comparison with a standard toxoid by protective units obtained after immunization (WILDFÜHR 1959, KRAUS 1924).

The major antigenic determinants and the toxophore groups of the toxin molecule, which are essential for the toxic effect and the membrane binding, are known nowadays (WELLHÖNER *et al.* 1982, VÖLCKERS 1986, AHNERT-HILGER *et al.* 1983, MATSUDA *et al.* 1975, 1977, SHEPPARD *et al.* 1984, HELTING *et al.* 1977). The antibodies obtained have been mainly tested for their neutralizing capicities. AHNERT-HILGER *et al.* (1983) identified four groups of monoclonals: (1) anti-toxoid antibodies reacting to toxin, toxoid, the light chains, the fragment B, but not to the fragment C, and which are not neutralizing *in vitro* and *in vivo*, (2) neutralizing antibodies reacting to toxin, toxoid and the light chains, (3) monoclonal antibodies recognizing only toxoid, and (4) monoclonal antibodies reacting to toxin, toxoid,

the fragment C, but not the fragment B or the light chains. Neither as single antibodies nor as mixture were the latter and the monoclonal antibodies described by other groups an alternative to polyclonal antiserum to be used for therapeutic purposes. Therefore, human monoclonal antibodies have been studied (ZURAWSKI *et al.* 1978, BOYD *et al.* 1984), but also without success with regard to neutralizing effects.

Different monoclonal antibodies obtained after immunization of mice or from healthy persons should be characterized and tested for their suitability in enzyme immunoassays (EIA).

Materials and methods

Preparation and production of monoclonal antibodies: Monoclonal antibodies were produced after long-term immunization of mice and healthy persons as described by KÖHLER and MILSTEIN (1975). The antibodies can be purified by conventional methods from cell culture supernatants after having been transferred into mass culture. Prior ultrafiltration over a XM-300 membrane (AMICON, Ousterhoot, The Netherlands) permits the obtainment of a monoclonal antibody concentration (factor 10–20). The monoclonal antibodies are enriched by affinity chromatography on insolubilized protein A to a purity of more than 90%.

Conjugates: The human monoclonal antibody TTG2 to tetanus toxin, the anti-human IgG, and the anti-mouse IgG antibody from sheep were labelled with peroxidase in a molar ratio of 1 : 4 (IgG : POD) as described by WILSON and NAKANE (1978).

Detection of antibodies specific for tetanus toxin in the ELISA: Tetanus toxin was enriched by ultrafiltration and separated by gel chromatography on Sephacryl S–200 (column: 3 cm × 108 cm, flow rate: 3.8 ml/cm^2/h). The fractions were pooled according to the elution and toxicity profile.

Comparable toxins were kindly provided by Prof. BIZZINI (Institut Pasteur, Paris) and Prof. HABERMANN (Institute of Phamacology, Justus Liebig Univ., Giessen). Tetanus toxoid was purchased from SSW Dresden (FRG).

For the antigen-specific enzyme immunoassays, microtiter plates (LINBRO, Flow Lab, Hamden, USA) were coated with toxin or toxoid. The antigens were adjusted to a concentration of 5 mg/l in carbonate buffer, pH 9.6, and incubated overnight at 4 °C in the wells. Unbound antigen was removed by three washes with phosphate buffer, pH 7.2, containing 0.1% Tween 20 (SERVA, Heidelberg, FRG), and 0.3 mol/l NaCl (PT/NaCl). The antigen-specific monoclonal antibodies were detected after a 12-hour incubation of cell culture supernatants adjusted to an antibody concentration of 1.0 mg/l in PT/NaCl. The class- and species-specific conjugates were used to detect bound antibodies.

The substrate used for peroxidase was H_2O_2/o-phenylenediamine (FLUKA, Berlin, FRG). The reaction was stopped after 25 min by addition of 1.0 mol/l H_2SO_4, 0.05 mol/l Na_2SO_3.

The reaction volume of all the steps described was 50 µl. The absorbances were read bichromatically at 492 and 680 nm using a Multiscan MCC photometer (Labsystems Oy, Helsinki, Finland). For further characterization, three clones of each species were chosen which reacted to toxin only.

Immunoblotting: The toxin was separated with and without reductive cleavage by mercaptoethanol using electrophoresis in SDS-polyacrylamid gel (7.5%) as described by LAEMMLI (1970). The proteins were then immobilized by electrotransfer (25 mmol/l Tris/HCl pH 8.8, 20% v/v methanol; 250 mA; 4 °C, 16 h) on nitrocellulose (SCHLEICHER & SCHÜLL, Dassel, FRG). After the membrane was blocked by 5% w/v powdered skim milk in PBS, the antibodies were incubated (final concentration about 1.0 mg Ig/l) on nitrocellulose strips overnight at 4 °C. Bound antibodies were detected by class-specific conjugates. The substrate used was diaminobenzidine/H_2O_2 (in 0.1 mol/l Tris/HCl pH 7.6; 0.1% w/v $NiCl_2$).

Determination of dissociation constants: Dissociation constants were detected on toxin-coated plates (5.0 mg/l) according to FRIGUET *et al.* (1985). The concentration for a half-maximum binding was determined in preliminary tests using class-specific conjugate. Increasing amounts of toxin (1.0×10^{-11} to 5×10^{-6} mol/l) were used for incubating the antibody in the selected concentration (16 h, 4 °C). The mixture was then transferred to the coated plates, and unbound antibodies were detected as described above. The results were calculated by regression analysis for the reciprocal concentrations and the respectively transformed absorbances (A_0/A_0-A_n; $A_0 = A_{492}$ without free antigen, $A_n = A_{492}$ in the free antigen concentration "n")

Two-site binding assays: Plates were coated with murine monoclonal antibodies for 16 h at 4 °C at 5.0 mg/l. This was followed by a 120-min reaction to tetanus toxin or toxoid in a concentration between 10 mg/l and 0.15 µg/l. In the second step, the human monoclonal antibodies, adjusted to a concentration of 0.25 mg/l were incubated for another 2 h and detected using the above-described species and class-specific conjugate in a third incubation of 60 min. For analysis we determined the lower detection limits or the recovery (20fold determinations for each concentration or blank value) of the two antigens.

Results

We have chosen three of 12 human and also three of 6 murine monoclonal antibodies recognizing tetanus toxin and not toxoid in ELISA for further investigation. Four antibodies (2F7, 3G4, 5B2, und TTG3) reacted in Western blot to the reduced and the nonreduced tetanus toxin. For these antibodies the epitope can be expected on the heavy chain of the toxin (Fig. 1). The two other human monoclonal antibodies TTG2 and TTG9 did not recognize the whole molecule in the Western blot. Only after reductive cleavage does the TTG2 antibody bind to the light chain of the toxin.

Investigations of the dissociation constant for the murine antibody produced values between 3.91×10^{-9} and 8.97×10^{-10} mol/l. There were two human antibodies (TTG2 and TTG3) where the constants were found to be about 100 times lower (Tab. 1). The correlation coefficients between the reciprocal concentrations and the transformed absorbances found in linear regression were in each case higher than 0.99.

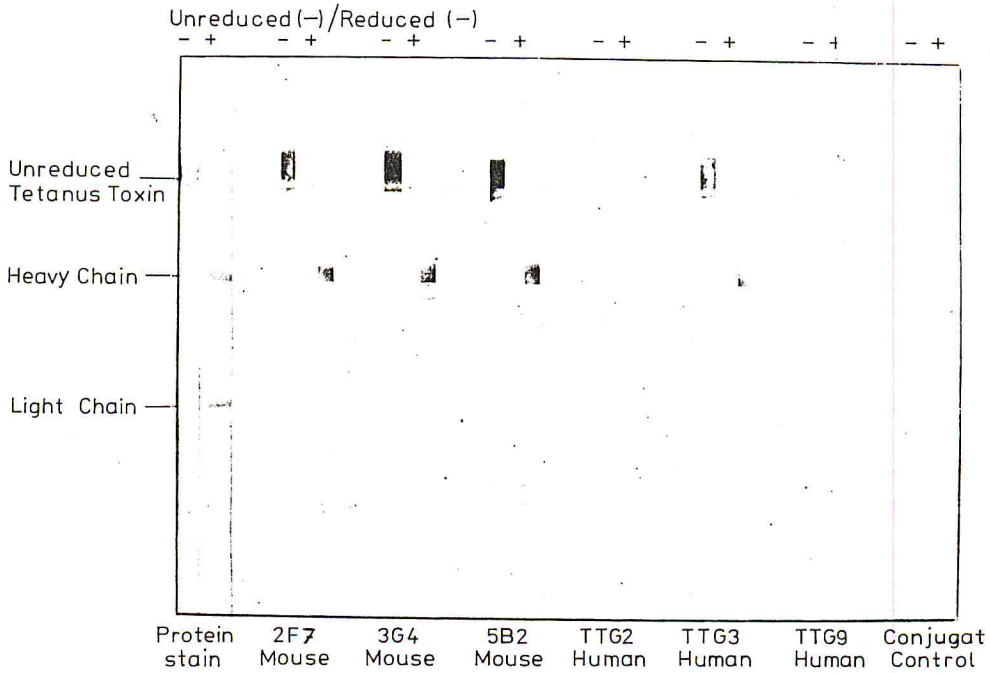

Fig. 1
Western blot of the human and murine monoclonal antibodies on reduced and non-reduced tetanus toxin

Table 1
Dissociation constants (K_D) for the murine and human monoclonal anti-tetanus antibodies

Species clone	K_D [mol/l]	Regression line	Correlation coefficient
Mouse			
2F7	3.91×10^{-9}	$y = 3.91 \cdot 10^{-9}x + 1.6$	0.998
3G4	4.39×10^{-9}	$y = 4.39 \cdot 10^{-9}x + 1.4$	0.997
5B2	8.79×10^{-10}	$y = 8.97 \cdot 10^{-10}x + 2.1 \cdot 10^{-6}$	0.990
Human			
TTG2	1.37×10^{-11}	$y = 1.37 \cdot 10^{-10}x + 1.1$	0.999
TTG3	8.48×10^{-12}	$y = 8.48 \cdot 10^{-12}x + 0.9$	0.998
TTG9	1.18×10^{-10}	$y = 1.18 \cdot 10^{-10}x + 1.1$	0.999

For the two-site binding assay to detect tetanus toxin, only one combination turned out to be useful after preliminary studies, ie the murine monoclonal antibody 2F7 bound to solid phase and the human monoclonal used as second antibody in conjugate. This combination of two monoclonal antibodies of different species finally permitted the detection of tetanus toxin up to a lower detection limit of 1.2 ng/ml (Fig. 2). Only 2,500 to 5,000 fold concentrations of tetanus toxoid led to detectable reactions by using this antibody combination.

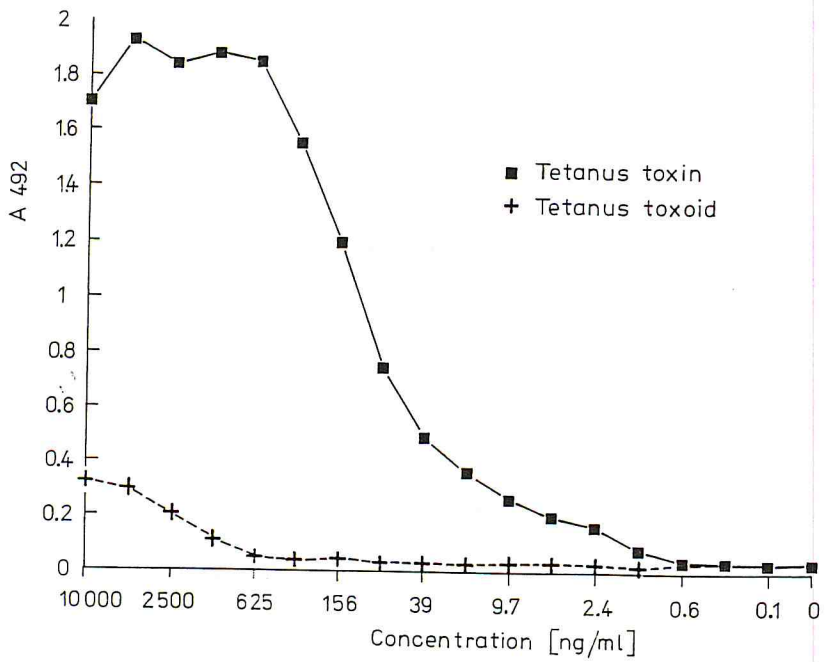

Fig. 2
Calibration curves of the tetanus EIA for tetanus toxin and tetanus toxoid showing to good discrimination between them and the upper detection limit for the tetanus toxin

Discussion

The majority of the monoclonal antibodies described recognize the heavy chain of tetanus toxin. None of the antibodies exhibited neutralizing effects in experiments *in vivo* (data not shown), whereas, on the other hand, AHNERT-HILGER *et al.* (1983), SHEPPART *et al.* (1984) and VOLK *et al.* (1984) had shown that monoclonal antibodies, if they are neutralizing, generally react to the C fragment of the heavy chain of the toxin. Therefore, we may assume, on the basis of the Western blot results, that the antibodies 2F7, 3G4, 5B2 and TTG3 recognize an epitope on the fragment B. This has also been confirmed by the results obtained by GORETZKI and HABERMANN (1985), who also classified the fragment B ($\beta 2$) as very immunogenic.

Antibodies reacting to the conformation determinants of the toxin have not yet been described, although a large number of antitetanus toxin antibodies are known. MIZUGUCHI *et al.* (1984) were the only ones who observed monoclonal antibodies which had "clear line precipitates" in immunodiffusion tests.

The murine monoclonal 2F7, which had been used for the development of the tetanus toxin EIA, reacts to tetanus toxin with a dissociation constant of 3.91×10^{-9} l/mol. The potential epitope is on the heavy chain of the toxin so that after blotting the antibody was detectable on the non-reductively split and the reductively split antigen. The human monoclonal antibody TTG2, which was used as secondary antibody, has a dissociation constant of 8.48×10^{-12} l/mol. This antibody recognizes an epitope which is present on the native protein only, but not on the SDS-treated one. This epitope is destroyed in such a way that this method can not be used to assign the epitope to the respective chain, so that the epitope has to be described as a conformation determinant. The dissociation constants for all the antibodies tested were in the ranges described by other authors (see above).

Investigations on the suitability of antibodies for EIA revealed, however, that four of the six antibodies react to toxin epitopes which only exist if toxin is bound to solid phases (polystyrene or nitrocellulose). This is the reason why only two of the antibodies were suitable for the development of the EIA (FRIGUET *et al.* 1984). In the two-site binding assay for the detection of tetanus toxin, the combination of monoclonal antibodies of two species described produced a lower detection limit of 1.2 µg/l, which is acceptable for EIA. Only 2,500 to 5,000 fold concentrations of tetanus toxoid lead to detectable reactions. It cannot be excluded, however, that the low amounts of toxin present in every toxoid are detected in the EIA.

The EIA presented here could therefore be used to control vaccines and to produce toxin for the preparation of vaccines.

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