Immunogenicity Test of Tetanus Component in Adsorbed Vaccines by Toxin Binding Inhibition Test

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Samples from 20 lots of diphtheria-tetanus (adult use dT) vaccine and from 20 lots of diphtheria-tetanus-pertussis (DTP) vaccine were used to standardize and validate the in vitro toxin binding inhibition (ToBI) test for the immunogenicity test of the tetanus component. The levels of tetanus antitoxin obtained by ToBI test were compared to those obtained using the toxin neutralization (TN) test in mice routinely employed to perform the quality control of the tetanus component in adsorbed vaccines. The results ranged from 1.8 to 3.5 IU/ml for dT and 2 to 4 IU/ ml for DTP by ToBI test and 1.4 to 3 IU/ml for dT and 1.8 to 3.5 IU/ml for DTP by TN in mice. These results were significantly correlated. From this study, it is concluded that the ToBI test is an alternative to the in vivo neutralization procedure in the immunogenicity test of the tetanus component in adsorbed vaccines. A substantial refinement and a reduction in use of animals can be achieved.

Key words: diphtheria-tetanus vaccine - diphtheria-tetanus-pertussis vaccine - toxin binding inhibition test - tetanus - quality control - seroneutralization

In the last decade, a big effort has been made to attempt replacement and reduction of animals in the development and control of biological products, mobilizing research scientists, manufacturers and regulators such as World Health Organization (WHO 1995, Sesardic & Hendriksen 1999). Besides ethical reasons, the replacement of challenge tests in animals by in vitro tests to control of biological products presents an additional impact for developing countries, as it is difficult to obtain quality of laboratory animals and adequate environmental conditions. Therefore, several in vitro serological assays for titration of tetanus antitoxin have been developed, such as, passive and indirect haemagglutination (Peel 1980, Gupta et al. 1984, Huet et al. 1992), several versions of enzymatic linked immunosorbent assay (ELISA) (Melville-Smith et al. 1983, Gentili et al. 1985, Simonsen et al. 1987, Esparza & Kissel 1992, Gupta & Siber 1994) and the toxin binding inhibition (ToBI) test (Hendriksen et al. 1988, 1989a). In this later test, the seroneutralization is followed by an ELISA which detects the free toxin into the toxin-serum mixtures. The ToBI test was first developed for determining antitoxin titres to tetanus in human sera (Hendriksen et al. 1988). Recently, it has also been proposed as an alternative assay to the in vivo challenge test for the potency control of tetanus toxoid vaccines (Hendriksen et al. 1991, WHO 1995).

In the present report, we describe the standardization and validation of the ToBI test to control the immunogenicity of the tetanus component in diphtheria-tetanus (adult use dT) and diphtheria-tetanus-pertussis (DTP) vaccines produced in our Institute. This control is routinely performed by a toxin neutralization (TN) test in mice according to the Minimum Requirement of Production and Control from the Brazilian Ministery of Health (Secretaria de Vigilância Sanitária 1998, Farmacopéia Brasileira 2000).

MATERIALS AND METHODS

Animals - Following the methodologies described by the Secretaria de Vigilância Sanitária (1998), guinea pigs weighing 450-550 g for immunization and BALB/c mice weighing 18-20 g for the TN test were obtained from the Butantan Institute Facilities, São Paulo, Brazil.

Vaccines - Twenty licensed lots of dT vaccine containing 4 Lf/ml of diphtheria toxoid, 15 Lf/ml of tetanus toxoid and 20 licensed lots of DTP vaccine containing 15 Lf/ml of diphtheria toxoid, 15 Lf/ml of tetanus toxoid and 32 OU/ml of whole cell of *Bordetella pertussis*, adsorbed onto 0.5 mg/ml of aluminum hydroxide routinely produced by Division of Bacterial Products at Butantan Institute, were used to immunized the animals.

Standard tetanus toxin, anatoxin and antitoxin - Tetanus toxin (1,000 Lf/ml) and anatoxin (1,520 Lf/ml), prepared in the Bacterial Vaccine Section at the Butantan Institute, was used for the in vivo TN test and for the ToBI test, respectively. Standard tetanus antitoxin (1,000 IU/vial) was obtained from the National Health Control Authority (INCQS-Fiocruz, Rio de Janeiro, Brazil) and diluted at 10 UI/ml in PBS with 50% of glycerol was stored at -20ºC.

Immunization of guinea pigs - Six guinea pigs were injected subcutaneously with half of the immunizing dose used for vaccinating human (0.75 ml). After six weeks the

animals were bled by cardiac puncture and the sera were inactivated (56°C/30 min) and stored at -20° C.

In vivo TN test - This test was carried out according to the Brazilian Ministry of Health (Secretaria de Vigilância Sanitária 1998). The serum pool, from at least four immunized guinea pigs, was prepared by mixing equal volumes of each collected serum. The antitoxin titre was determined by mixing 1 ml of each serum pool, diluting the serum pool to a L+/10/50 dose of tetanus toxin defined as a dose of toxin in 1 ml of PBS able to neutralize 0.1 IU of standard antitoxin, killing mice with 18-20 g within 96 h. After incubation at 37°C for 45 min, 0.75 ml of the mixture was subcutaneously injected in 10 normal mice. A control test with a mixture containing the toxin (L+/10/50) and three dilutions of the standard antitoxin were tested in parallel, also using 10 mice per dilution. The animals were observed for four days for tetanus symptoms and deaths. The lethal dose 50 (LD50) value of the tested sample was determined by Probit analysis. The potency of the tetanus component was calculated by A/BxC, where A was the effective dose 50 (ED50) of standard tetanus antitoxin, B was the LD50 of tested serum and C the IU/ml of standard tetanus antitoxin.

ToBI test - This test was performed as described by Hendriksen et al. (1989a) by employing tetanus anatoxin instead of tetanus toxin.

Peroxidase-labeled antitetanus IgG F(ab')$_2$ - Purified antitetanus F(ab')$_2$ fragments were prepared by enzymatic digestion from hyperimmunized horse serum in the Technological Development and Production Division at the Butantan Institute. The conjugate peroxidase-glutaraldehyde-F(ab')$_2$ was prepared as described by Avrameas and Ternynck (1971). The produced conjugate, at a 1:2000 dilution, was able to detect 0.062 Lf/ml of tetanus anatoxin.

Guinea pig serum pool dilutions - A round-bottom polystyrene microplate was blocked with 200 µl/well of skimmed milk at 1% during 90 min at 37°C. After blocking, the microplate was washed with buffer A (saline pH 7.2 and 0.05% of Tween-80) and dried. The serum pool samples were diluted in PBS from 1:2 to 1:1024 in a volume of 100 µl in duplicate. In parallel, a curve of standard tetanus antitoxin was made to plot the results of unknown serum samples. The standard antitoxin was diluted from 10 to 0.156 IU/ml in two horizontal columns with 100 µl/well. A volume of 100 µl of tetanus anatoxin (1 Lf/ml) was added in all wells. The positive control was made in an horizontal column with anatoxin and PBS (100% absorbance) and the negative control in the subsequent column with PBS only. The microplate was incubated during 60 min at 37°C and then overnight at 4°C.

Coated plate for ELISA assay - A microplate of polyvinyl chloride for immunoassay was coated with 100 µl of carbonate buffer pH 9.6 containing 20 µg/ml of F(ab')$_2$ of antitetanus IgG F(ab')$_2$ fragments. After incubation overnight at 4°C, the microplate was washed three times with buffer A and blocked with 200 µl of skimmed milk at 1% in PBS pH 7.2 during 60 min at room temperature and then washed and dried. One hundred microliters of the serum-anatoxin mixtures were transferred to each corresponding well of coated microplate and incubated for 90 min at 37°C.

After three washings with buffer A and drying, 100 µl of the diluted peroxidase-conjugate (1:2000) in PBS were then added in all wells and the microplate incubated again for 60 min at room temperature in the dark. After three washings with buffer A, 100 µl of the substrate (1.7% of 25 mM Tetramethyl-Benzidine, 0.03% of H_2O_2 in 11mM sodium acetate buffer, pH 5.5) was added as described by Hendriksen et al. (1989a). After 15 min in dark, the reaction was stopped by the addition of 2M H_2SO_4. The absorbance was measured at 450 nm using a Multiskan EX/Labsystem automatic plate reader. The maximum absorbance (Ab_{100}) were obtained from the wells with standard anatoxin only. These values were equal to 1.6 with a standard deviation of ± 0.005. The Ab_{50} was determined as half of the Ab_{100} average, corresponding thus to wells with approximately 50% of neutralized anatoxin. The serum samples which values were near to Ab_{50} were plotted in a standard antitoxin curve. The antitoxin titre of tested serum pools was calculated by the Ab_{50} value multiplied by the inverse of the corresponding dilution and expressed in IU/ml. The calculation was made using the software Multicont (Multiskan Transmit Software revision 4.5).

Statistical analysis - The Kruskal-Wallis nonparametric tests were employed to evaluate the homogeneity among the repetitions of tests per lot of vaccines and among the results of lots. The Spearman's correlation coefficient was used to compare the relationship between the ToBI test and the in vivo TN test. Statistical analysis were performed using the Statistical Package of Social Sciences software (1997).

RESULTS

ToBI test was employed to determine the levels of tetanus antitoxin in pooled sera from guinea pigs immunized with different lots of dT and DTP vaccines. These results were compared to the in vivo TN test used routinely to perform the potency test of tetanus component in adsorbed vaccines.

Fig. 1 represents the standard curve of the standard tetanus antitoxin, ranging from 0.156 to 10 IU/ml and mixed with 1 Lf/ml of tetanus anatoxin. The lowest concentration of tetanus antitoxin able to neutralize 1 Lf/ml of anatoxin was 0.156 IU/ml in the ToBI test.

Tables I and II show the potency results of the tetanus component of different lots of dT and DTP vaccines obtained by ToBI and in vivo neutralization tests. The titre values were ranged from 1.8 to 3.5 IU/ml for dT and 2 to 4 IU/ml for DTP by ToBI test and 1.4 to 3 IU/ml for dT and 1.8 to 3.5 IU/ml for DTP by TN test in mice. The ToBI/in vivo ratio of mean titre was 1.24 (± 0.096) for dT and 1.14 (± 0.07) for DTP. The statistical analysis applied to evaluate the reproducibility of the results did not detect significant difference in seven and three separate experiments of in vitro and in vivo tests, respectively, for all lots of dT or DTP analyzed. The results obtained by ToBI test presented a high homogeneity according to the Kruskal-Wallis test for each lot of dT and DTP vaccines analyzed with H = 1.367 (p = 0.968) and H = 0.487 (p = 0.998), respectively. Similar results was also found for the in vivo neutralization test for the same lots evaluated with H = 0.232
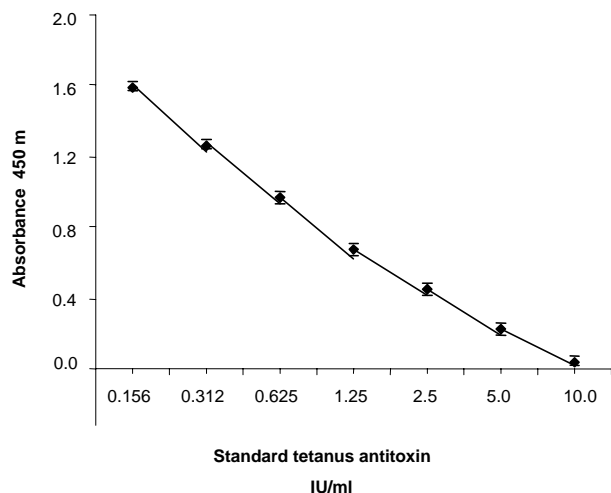
Fig. 1: different concentrations of standard tetanus antitoxin were mixed with 1 Lf/ml of tetanus anatoxin. The results were plotted as mean with bars indicating ± SD from 20 independent tests.

TABLE I

Tetanus component potency of diphtheria-tetanus vaccine by in vitro and in vivo neutralization tests

Vaccine	Tetanus antitoxin (IU/ml)		Ratio in vitro/in vivo
Lots	ToBI	L+/10/50	ToBI/in vivo
A	2.30(±0.1)	1.80(±0.1)	1.28
B	2.50(±0.3)	2.25(±0.2)	1.11
C	3.00(±0.4)	2.60(±0.1)	1.15
D	2.00(±0.2)	1.80(±0.4)	1.11
E	3.50(±0.2)	3.00(±0.1)	1.17
F	3.00(±0.1)	2.50(±0.3)	1.20
G	3.00(±0.2)	2.50(±0.2)	1.20
H	2.50(±0.1)	2.00(±0.1)	1.25
I	2.00(±0.2)	1.50(±0.3)	1.33
J	2.50(±0.1)	2.00(±0.2)	1.25
K	2.00(±0.3)	1.40(±0.2)	1.43
L	2.00(±0.1)	1.40(±0.2)	1.43
M	2.00(±0.2)	1.80(±0.2)	1.11
N	2.00(±0.1)	1.80(±0.1)	1.11
O	2.00(±0.4)	1.50(±0.1)	1.33
P	2.50(±0.2)	2.00(±0.1)	1.25
Q	2.00(±0.1)	1.60(±0.2)	1.25
R	1.80(±0.1)	1.40(±0.2)	1.28
S	2.50(±0.2)	2.00(±0.3)	1.25
T	2.50(±0.3)	2.00(±0.3)	1.25
Mean (±)	2.38 (0.46)	1.94 (0.44)	1.24 (0.096)
Kruskal-Wallis (H)	123.562 p = 0.000	54.353 p = 0.000	

The result of each lot of vaccine represents the mean ± SD from 7 repeated experiments for the toxin binding inhibition (ToBI) test and 3 repeated experiments for the in vivo test. The Kruskal-Wallis test among repetitions for ToBI test was H = 1.367 (p = 0.968) and for the in vivo test was H = 0.232 (p = 0.891). These results are not significantly different. The results inter lots are significantly different.

TABLE II

Tetanus component potency of diphtheria-tetanus-pertussis vaccine by in vitro and in vivo neutralization tests

Vaccine	Tetanus antitoxin (IU/ml)		Ratio in vitro/in vivo
Lots	ToBI	L+/10/50	ToBI/in vivo
A	3.20(±0.2)	2.80(±0.2)	1.14
B	2.50(±0.1)	2.25(±0.2)	1.11
C	3.00(±0.3)	2.60(±0.1)	1.15
D	2.00(±0.1)	1.80(±0.2)	1.11
E	3.50(±0.4)	3.00(±0.1)	1.17
F	3.00(±0.2)	2.50(±0.3)	1.20
G	2.50(±0.1)	2.00(±0.1)	1.25
H	2.00(±0.1)	2.00(±0.2)	1.00
I	2.00(±0.1)	2.00(±0.2)	1.00
J	4.00(±0.4)	3.50(±0.1)	1.14
K	2.50(±0.3)	2.00(±0.3)	1.25
L	2.80(±0.1)	2.60(±0.1)	1.08
M	2.00(±0.2)	1.80(±0.4)	1.11
N	3.50(±0.1)	3.00(±0.1)	1.17
O	3.00(±0.2)	2.50(±0.2)	1.20
P	2.50(±0.1)	2.00(±0.1)	1.25
Q	3.00(±0.2)	2.60(±0.3)	1.15
R	2.00(±0.1)	1.80(±0.1)	1.11
S	2.00(±0.2)	1.80(±0.2)	1.11
T	2.50(±0.3)	2.10(±0.1)	1.19
Mean (±)	2.68 (0.59)	2.33 (0.49)	1.14 (0.07)
Kruskal-Wallis (H)	130.072 p = 0.000	53.814 p = 0.000	

The result of each lot of vaccine represents the mean ± SD from 7 repeated experiments for the toxin binding inhibition (ToBI) and 3 repeated experiments for the in vivo test. The Kruskal-Wallis test among repetitions was H = 0.487 (p = 0.998) for the ToBI test and H = 1.133 (p = 0.567) for the in vivo test. These values are not statistically different. The results inter lots are statistically different.

(p = 0.891) for dT and H = 1.133 (p = 0.567) for DTP. However, a high heterogeneity of results was observed among the lots for both vaccines, by ToBI as well as by TN in mice.

Fig. 2 shows the distribution of tetanus antitoxin levels obtained by ToBI and in vivo neutralization tests for all lots of dT and DTP analyzed. The Spearman's correlation coefficient was also calculated demonstrating an excellent correlation of these tests for each lot of dT (r = 0.940 and p = 0.000) and DTP (r = 0.951 and p = 0.000) studied.

DISCUSSION

The principle of the ToBI test is related to the in vivo TN test procedure and is based on the detection of free toxin in toxin-serum mixtures, using an ELISA instead of live animals. The only difference between the ToBI test and the in vivo TN test is the way in which the free toxin is assayed, e. g. by ELISA or in a mouse assay (Hendriksen et al. 1989a).

Butantan Institute is the major Brazilian manufacturer of bacterial adsorbed vaccines since 1967 and supplies in part the National Program of Immunization organized by
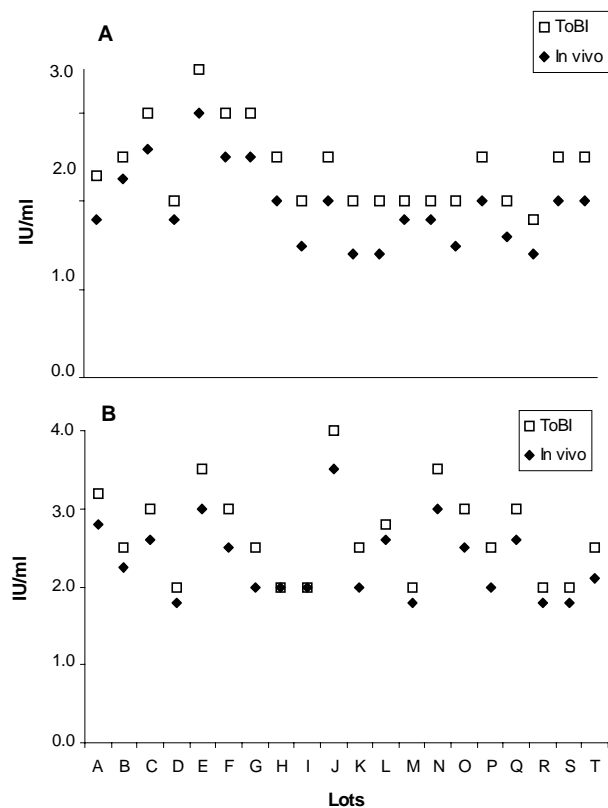
Fig. 2: distribution of tetanus antitoxin levels obtained by in vivo and in vitro tests per lot. Spearman's correlation coefficient between in vivo and in vitro results (Tables I and II) demonstrated r = 0.940 (p = 0.000) for the diphteria-tetanus vaccine and r = 0.951 (p = 0.000) for the diphiteria-tetanus-pertussis vaccine. ToBI: toxin binding inhibition

The results obtained by ToBI test were very similar to the results obtained using the in vivo TN test. The arithmetic means of in vitro/in vivo ratio obtained by ToBI and TN in mice for both vaccines have shown good correspondence. The Kruskal-Wallis test showed high homogeneity between the ToBI test and the TN in mice among the results for each lot of vaccine analyzed. The heterogeneity of results among the lots studied could, at least in part, be due to the individual ability of guinea pigs to respond to the vaccine inoculation, but also by some difference in the concentration of components during vaccine formulation. Hendriksen et al. (1989a) also reported a significant correlation of these methods assaying tetanus and diphtheria antitoxin in human sera.

Apart from the similarity of results found by in vitro and in vivo tests, the ToBI test offers distinct advantages in relationship to TN in mice such as: the use of anatoxin instead of toxin, no requirement of a large number of animals, its ease to perform and the immediacy of results.

Our results lead to the conclusion that the substitution of the in vivo TN test by the ToBI test is pertinacious for assaying tetanus immunogenicity in the quality control of dT and DTP vaccine production.

These results will contribute to the establishment of alternative and more refined tests for the quality control of adsorbed vaccines by the National Control Authorities in Brazil. Further, they can also stimulate other manufacturers from developing countries to perform in vitro tests for the quality control of their vaccine production. Thus, leading amplify the network of knowledge to replacement, reduction and refinement, the so-called 3Rs (Sesardic & Hendriksen 1999), of animals in control of biological products.

Brazilian Ministry of Health. Although this Institute has good conditions for animal facilities, the current protocols for biological control for in-process and final production of vaccines are expensive, toilsome and the release of final results requires several days. Its production of dT and DTP vaccines shows an excellent consistence of the quality of vaccine produced. In this context, the set of results presented herein and analyzed statistically prove that the ToBI test is precise and reproducible when compared with the in vivo TN test currently used. Other authors also have related that this in vitro test demonstrates to be specific, sensitive, reproducible and the results less variable in relation to in vivo TN tests in mice (Hendriksen et al. 1988, 1991).

The ToBI test has been successfully used to determine diphtheria antitoxin in human and animal sera (Hendriksen et al. 1989b, Hong & Hendriks 1999, Marcovistz et al. 2002).

The essential difference between the ToBI test developed by Hendriksen et al. (1988) and that standardized by us was the use of tetanus anatoxin instead of tetanus toxin. This was possible because the detoxification of the toxin does not modify its antigenic epitopes and can be handled without risk of health for the staff.

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