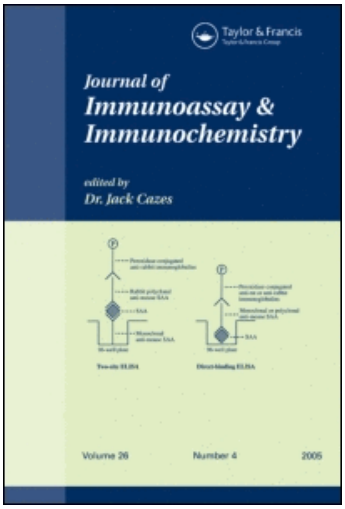

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Solid Phase Enzyme Immunoassays of Pertussis Toxin Using Peroxidase or Biotin Labelled Antibodies

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SOLID PHASE ENZYME IMMUNOASSAYS OF PERTUSSIS TOXIN USING PEROXIDASE OR BIOTIN LABELLED ANTIBODIES.

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ABSTRACT

The quantitative determination of pertussis toxin (PT) is generally estimated by biological tests which are time-consuming, cumbersome and unsuitable for simultaneous testing of a large number of samples. The present work describes a rapid and sensitive ELISA procedure allowing PT assay based on a sandwich technique amplified via avidin-biotin interaction. As low as 0.1 ng of PT in 0.1 ml sample could be detected by the procedure described.

KEY WORDS: Pertussis toxin, Enzyme immunoassay, Avidin-biotin amplification.

INTRODUCTION

Among the toxins produced by Bordetella pertussis the causative agent of whooping cough, pertussis toxin (PT)

(1,2) also known as lymphocytosis promoting factor, histamine-sensitizing factor, islet-activating factor and pertussigen (3,4,5) appears as the major toxic protein. PT injected into experimental animals, is lethal and induces hypoglycemia, leukocytosis and lymphocytosis as well as sensitization to the lethal effects of histamine. The toxin has also adjuvant effects and is a potent immunogen for protection of mice against challenge with B. pertussis (6). PT was also shown to facilitate progesterone induced maturation of Xenopus oocyte (7). The toxin has been purified (8) and it was shown to be constituted by five subunits (9) and in conformity with the A (active fragment or protomer) - B (binding moiety) model of various bacterial toxins (10,11). Subunit S1 (M_r ≈ 28,000) is the A protomer which triggers the physiologic and cellular action of PT by catalysing the transfer of ADP - ribose from NAD to a 41 KDa protein in various cells (12) including rat brain cells (13). This protein is one of the subunits of the guanine nucleotide regulatory protein (Ni) that is responsible for inhibition of adenylate cyclase (14, 15, 12) and thereby is a useful probe for studying the mechanisms involved in coupling between receptors and adenylate cyclase (16).

In spite of the great importance of PT in immunological, biochemical and biological studies, no simple, rapid and accurate techniques for a quantitative estimation of the toxin are available. A variety of *in vivo* assays have been described based on promotion of lymphocytosis, histamine sensitization and potentiation of insulin secretion (17, 18, 5) or on the effect on chinese hamster ovary cells (19). These assays have obvious disadvantages. They are expensive, time-consuming and require a number of animals for appropriate statistical evaluation.

Recently, (20) an enzyme-linked immunosorbent assay (ELISA) of PT was developed, based on the specificity of this toxin for haptoglobin (21). This haptoglobin-ELISA method showed good correlation with leukocyte promoting activity *in vivo*. One ng of PT could be detected by this technique. We report in this article a systematic comparative study of various ELISA methods using antigen or antibody coated plates or beads, with or without amplification systems in order to determine optimal conditions for PT immunoassay. We describe a rapid and easy enzyme immunoassay allowing the detection of as low as 0.1 ng of PT in test samples.

MATERIALS AND METHODS

Reagents

Horseradish peroxidase (Grade 1) was obtained from Boehringer (Mannheim, FRG), gelatin AFNOR 59 002 from Rousselot-Kuhlmann (FRG). Tween 20,D(+) biotin, dimetylformamide and 25% glutaraldehyde aqueous solution were purchased from Merck (Darmstadt, FRG), bovine serum albumin, hydroxylapatite, Magnogel and DEAE-Trisacryl-M from Industrie Biologique Française (Villeneuve-La-Garenne, France). Avidin, 2-4-6 trinitrobenzene sulfonic acid, and o-phenylenediamine dichloride were obtained from Sigma chemical Co (Saint-Louis, Missouri, USA) redistilled glycerol and 30% hydrogen peroxide from Prolabo (Paris, France). N-N-carbonyl diimidazole and N-hydroxysuccinimide were obtained from Aldrich Co (Milwaukee, Wisc., USA). D-carbonyl ^{14}C-biotin was purchased from Amersham (Buckinghamshire, U.K.). AH Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Microtitration plates with 96, 300 µl flat bottomed wells were purchased from Nunc (Roskilde, Denmark). Polystyrene balls 6.5 mm in diameter were provided by Precision Plastic Balls Inc.

(Chicago, Ill, USA). Rabbit IgG were obtained from Miles Laboratories (Kankakee, Ill, USA).

Preparation of Pertussis Toxin

The toxin was purified from the culture supernatant of B. pertussis 509-22 (Institut Pasteur Production, Le Vaudreuil, France). Bacterial culture and concentration of culture supernatant fluid were carried out as reported previously (5). PT was purified by successive column chromatography on hydroxylapatite (22) and haptoglobin-AH sepharose 4B (23). Purified PT was concentrated on Amicon PM 10 membrane (Amicon, Lexington, Mass, USA), dialyzed against 150 mmol/l NaCl containing 10 mmol/l phosphate buffer pH 7.2 (PBS) and kept at -20°C. Protein concentration was measured after dialysis (24) using bovine serum albumin as a standard.

Biological assay of Pertussis Toxin

The biological activity of PT was determined by the measurement of its leucocytosis-promoting activity three

days after intraperitoneal injection of appropriate dilutions of the toxin into 18-20 g swiss mice. As defined in a previous work (5) one unit of leucocytosis promoting activity is that amount of toxin which increases two-fold the number of white blood cells per mm^3. One unit of purified PT corresponded to about 100 ng of protein.

Preparation of Anti-pertussis Toxin IgG

New Zealand rabbits were hyperimmunized with purified PT. The animals were injected intradermally into the back with 0.5 ml of PBS containing 50 µg of non detoxified PT emulsified in 0.5 ml complete Freund adjuvant (Difco Laboratories, Detroit, Mich, USA). They were then injected on days 14, 28, 42, 52 and 84 with 50 µg of the same antigen emulsified in 0.5 ml of incomplete Freund adjuvant and bled 3 weeks after the last injection. The IgG fraction was obtained by fractionation of the immune serum on DEAE-Trisacryl-M column (25).

Protein Biotinylation

Biotinyl-N-hydroxysuccinimide (BNHS) was used for introducing biotin moieties into bovine serum albumin and

anti PT-IgG respectively. BNHS was prepared (26) by using N-N-carbonyl diimidazole to form an intermediate biotin derivative which was subsequently converted into the N-hydroxysuccinimide ester by the addition of N-hydroxysuccinimide. BNHS was kept dessicated under vaccum until use. Ten mg of BSA or IgG were dissolved in 1 ml 100 mmol/l $NaHCO_3$ solution and mixed with 0.18 or 0.11 ml of 200 mmol/l BNHS solution in distilled dimethylformamide so that to obtain a BNHS amount of $3.6\ 10^{-5}$ moles for BSA and $2.2x10^{-5}$ for IgG.

These amounts were selected for the optimization of the number of biotin molecules linked per molecule of protein on the basis of 4 moles of BNHS per mole of free amino groups (61 in BSA and 90 in IgG) as reported previously (27). The reaction mixture was incubated at room temperature for 1 h and then dialyzed for 24 h at 4°C against several changes of PBS. After dialysis the free amino-groups in biotin substituted proteins were determined by using the 2-4-6 trinitro benzene sulfonic acid procedure (28). An equal volume of redistilled glycerol was added and the preparation was kept at -20°C until used. The percentage of biotinyled amino-groups for BSA and anti-PT IgG was 88.9% and 94.6% respectively.

Preparation of Anti-biotin Antibodies

Five hundred micrograms of biotinylated BSA in 0.5 ml of PBS were emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the back and footpads of New Zealand rabbits. Two consecutive injections of 0.5 mg biotinylated BSA with adjuvant were given subcutaneously at three week interval starting three weeks after the first immunization. The rabbits were boosted subcutaneously one month later with 1 mg of biotinylated BSA. Ten days after the last injection, the rabbits were bled.

Anti-biotin antibodies were isolated by batchwise, mixing 10 ml of the whole immune serum with 10 ml of an immunoadsorbent prepared as follows. Commercial rabbit IgG (7.3 mg) was bound to Magnogel by means of glutaraldehyde (29).

The immobilized IgG was then biotinylated by incubating the gel overnight at room temperature with 15 mg of BNHS dissolved in 1 ml of dimethylformamide and then adjusted to 10 ml with 100 mmol/l $NaHCO_3$ solution. Biotin binding was controlled by incubating an aliquot (50 µl) of the immunoadsorbent with peroxidase-labelled avidin. The

anti-biotin antibodies were desorbed from the immunoadsorbent by means of 0.2 N HCl-0.7 mol/l glycine buffer pH 2.8 and, then neutralized, concentrated and dialysed according to usual methods.

The affinity of the isolated anti-biotin IgG was determined by equilibrium dialysis against various concentrations (from 1.10^{-6} to $20\ 10^{-6}$ mol/l) of D-carbonyl ^{14}C-biotin (59 mCi/mmol) with an initial volume of 0.4 ml in each chamber of the dialysis cell. The antibody concentration was $1.5\ 10^{-6}$ mol/l. The solvent was 0.15 mol/l NaCl containing 0.010 mol/l phosphate buffer pH 7.4 and 0.25 g/l BSA. . The results were analyzed according to the law of mass action derived by Scatchard (30).

Preparation of Enzyme-conjugates

Peroxidase was coupled to rabbit anti-PT IgG and to rabbit anti-biotin antibodies by the one-step glutaraldehyde coupling procedure (31,32). Avidin was labelled with peroxidase as described elsewhere (27). After coupling, the preparations were stored at -20°C in 50%

redistilled glycerol. The amount of peroxidase used for conjugation was 2-fold that of IgG and avidin.

Enzyme Linked Immunosorbent Assay (ELISA)

a) Buffers

The following buffers were used: coating buffer: 150 mmol/l NaCl containing 10 mmol/l phosphate buffer (pH 7.2) ; washing buffer: coating buffer containing 0.1% Tween 20 ; incubation buffer: washing buffer containing 0.25% gelatin.

b) Direct ELISA

Microtitration plates were coated with aliquots (100 µl/well) of PT dilutions in coating buffer at concentrations ranging from 500 µg to 0.4 µg of toxin/l. The plates were covered and incubated at 37°C for 2 h and then at 4°C for 14 to 18 h. After coating the plates were washed three times with the washing buffer and 100 µl of peroxidase-labelled anti-PT IgG (5 mg/l) were added into the wells. The plates were then covered, incubated at 37°C for 1 h and washed three times. Peroxidase activity was determined by adding 200 µl of substrate solution per well.

This solution was composed of 50 mmol/l citrate buffer (pH 5.4) containing 1 g of o-phenylenediamine dichloride and 2 ml of 30% hydrogen peroxide per l. The enzyme reaction was stopped after 10 minutes by adding 50 µl of 3 N HCl to each well. Absorbance was read with a Multiskan photometer (Flow Laboratories Inc, McLean Va, USA) at 492 nm.

c) Sandwich ELISA

This test was performed by two different techniques based on coating microtitration plates or polystyrene balls with anti-PT IgG. The microtitration plate technique was carried out by coating each well with 100 µl of antibody solutions (5 mg/l) in coating buffer. The plates were covered and incubated at 37°C for 2 h and then at 4°C for 14 to 18 h. After coating, the plates were washed three times. PT was diluted from 500 µg to 0.4 µg per l in the incubation buffer and 100 µl of the solution were added to the wells. The plates were covered, incubated at 37°C for 2 h and washed three times. 100 µl of peroxidase-labelled anti-PT IgG (5 mg/l) were added to the wells. Subsequent steps were similar to those described above.

The polystyrene balls technique was performed by washing the balls in distilled water, drying at 37°C and coating with four different doses of anti-PT IgG (1,5, 10,

20 mg/l) as follows. One ml of each dilution of coating solution was placed in a glass tube containing three balls. The tubes were incubated for 2 h at 37°C and overnight at 4°C. The balls were then separated, placed individually in glass tubes and washed with three successive 5 ml portions of washing buffer. Each of the four series of the IgG coated balls were allowed to react with three different concentrations of PT (500, 50, 10 µg/l of incubation buffer) by incubating individual balls with 500 µl of PT solutions at 37°C for 2 h. The balls were then washed three times as described above. Each ball was then separately incubated for 1 h at 37°C in glass tubes containing 500 µl of peroxidase-labelled anti PT IgG (5 mg/l). Peroxidase activity was determined after adding 500 µl of substrate into each test tube. Enzyme reaction was stopped after 30 minutes by adding 60 µl of 6 N HCl. 400 µl aliquots of each tube were transferred into microtitration plate wells. Absorbance was determined with a Multiskan photometer at 492 nm.

d) Amplification systems using biotin - labelled IgG and peroxidase - labelled avidin or peroxidase - labelled anti-biotin antibodies.

Well coating with anti-PT IgG followed by incubation with PT were made according to the procedure described for

the sandwich technique. Thereafter, 100 µl of biotin - labelled anti-PT IgG (1 or 5 mg/l) were added into the wells. The plates were incubated at 37°C for 2 h and washed three times. Then were added either 100 µl of peroxidase - labelled avidin at two different concentrations (1 or 5 mg/l) or peroxidase - labelled anti-biotin antibodies at four different concentrations (1, 5, 10 or 20 mg/l). The plates were incubated at 37°C for 1 h and washed three times. Peroxidase activity was determined by adding 200 µl of substrate. Enzyme reaction was stopped after 10 minutes by adding 50 µl of 3 N HCl to each well. Absorbance was read at 492 nm.

RESULTS

For the five different procedures described in this work the detection limit of PT was defined as the amount of toxin expressed in nanograms corresponding to the mean of background values of the optical density of the peroxidase reaction plus three standard deviation values. The sensitivity of an assay is defined as the ability to differenciate significantly between small differences of

concentrations or contents. This sensitivity (discriminative capability) depends on various parameters, among them the slope of the calibration curve is the essential one. Thus, for the five different ELISA systems described here we compared the slopes of the linear portion of the dose-response curves reflected by optical density increase at 492 nm (Fig. 1, 2, 3). The slopes were calculated in the concentration range of 6 to 12 ng of PT (Table 1).

Direct ELISA

This technique was the simplest one among those devised in this work. Plate coating concerned only PT. The detection limit of the toxin (Fig 1, curve A) was ca. 4.5 ng. The slope value (0.03) was low (Table 1).

Sandwich ELISA without amplification

The solid phase coated with anti-PT IgG was either microtitration plates or polystyrene balls. The peroxidase

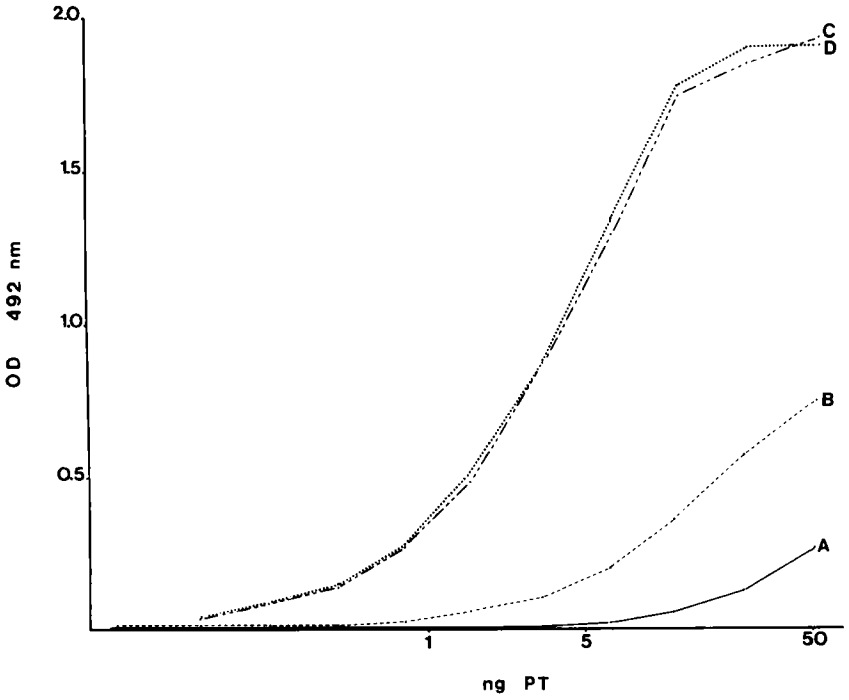

FIGURE 1

Immunoassay of pertussis toxin (PT) in microtitration plates using direct ELISA (A) or sandwich ELISA without amplification system (B) or with amplification by biotin/avidin system (C) or biotin/anti-biotin antibodies (D).
Curve A: wells coated with increasing PT concentrations followed by peroxidase-labelled anti-PT IgG (5 mg/l).
Curve B, C, D: wells coated with anti-PT IgG (5 mg/l) treated with increasing PT concentrations and followed by either peroxidase labelled anti-PT IgG, (5 mg/l) (B) or biotin-labelled anti-PT IgG, (5 mg/l) (C and D) and subsequent addition of either peroxidase-labelled avidin, (1 mg/l) (C) or peroxidase-labelled anti-biotin antibodies (10 mg/l) (D). Enzyme reaction time: 10 min.

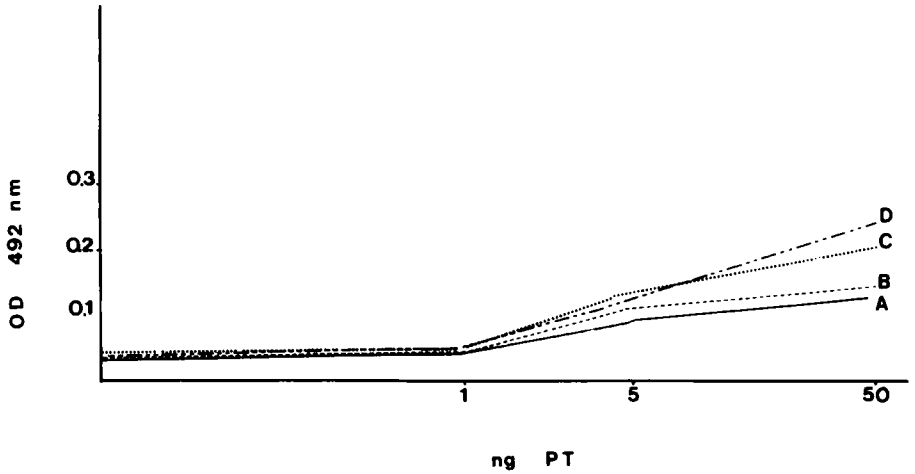

FIGURE 2
ELISA of pertussis toxin (PT) using polystyrene balls. The balls were coated with anti-PT IgG (1, 5, 10, 20 mg/l) respectively (curves A, B, C, D). The conjugate used was peroxidase-labelled anti-PT IgG (5 mg/l). Enzyme reaction time: 30 min.

conjugate used was at the same concentration in both procedures. For the former, optimal coating was obtained with 5 mg of anti-PT IgG/l. The detection limit was 0.7 ng of PT (Fig. 1, curve B) and the slope equal to 0.16 (Table 1).

With the ball procedure optimal coating was obtained for 10 mg of anti-PT IgG/l. The detection limit was 2 ng of PT (Fig. 2) with a slope value of 0.02 (Table 1).

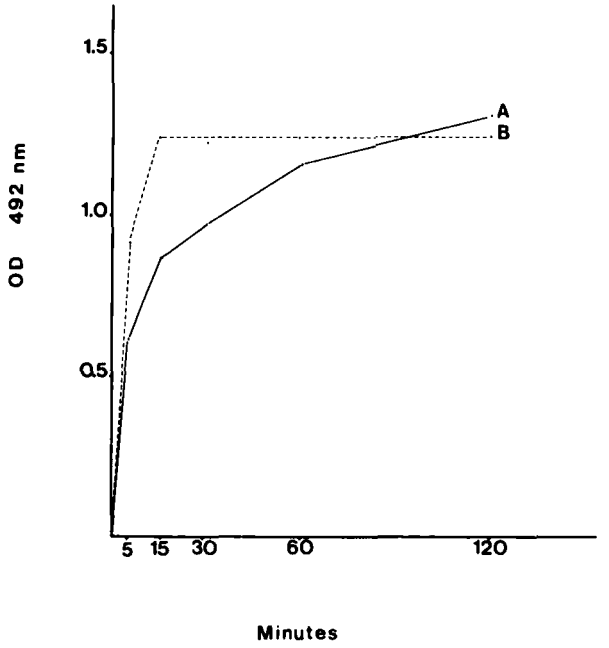

FIGURE 3

Enzyme-conjugate binding kinetics. The experimental conditions were similar in curves A and B except for peroxidase-conjugate step. The wells were coated with anti-PT IgG (5 mg/l) and successively filled with 50 µg of PT/l and then with biotin-labelled anti-PT IgG (5 mg/l). Peroxidase-avidin conjugate (1 mg/l) (curve A) or peroxidase labelled-anti-biotin antibodies (10 mg/l) (curve B) were added and allowed to react for 5, 15, 30, 60 or 120 minutes respectively before washing the plates and then adding H_2O_2 and o-phenylenediamine. Blank values were substracted from each OD value of reaction wells. Enzyme reaction time: 10 min.

TABLE 1

Comparison of the detection limit and sensitivity of the different ELISA procedures devised for PT assay.

Immunoassay(*)	PT detection limit(**)	Slope value(**)
Direct ELISA	4.5 ± 1	0.03 ± 0.005
Sandwich ELISA : Coated beads	2 ± 0.7	0.02 ± 0.005
Coated microplates (c.m.) without amplification	0.7 ± 0.005	0.16 ± 0.015
c.m. with amplification by biotin/ avidin system	0.1 ± 0.02	0.45 ± 0.020
c.m. with amplification by biotin/ anti-biotin system	0.1 ± 0.02	0.45 ± 0.020

(*) See Materials and Methods (**) See Results
All experiments were in triplicate.

Sandwich ELISA with amplification by biotin/avidin or biotin/anti-biotin systems

These amplifying systems were used in order to increase the number of peroxidase molecules linked to the anti-PT IgG. The steepness of the calibration curve with the biotin/avidin system technique was 3-fold higher (= 0.45)

than the sandwich ELISA (Table 1). The detection limit was ca. 0.1 ng which was therefore 7-fold less (Fig. 1, curve C). In this technique the best results were obtained by using 5 mg/l of biotinylated anti-PT IgG and 1 mg/l of peroxidase - avidin conjugate.

For the biotin/anti-biotin system, the results were identical to the biotin/avidin system as regards detection limit (Fig. 1, curve D) and sensitivity (Table 1). Optimal results were obtained by using 5 mg/l of biotinylated anti-PT IgG and 10 mg/l of anti-biotin antibodies. The affinity of these antibodies determined by equilibrium dialysis was relatively weak ($K_A \simeq 0.5 \times 10^6\ M^{-1}$).

Enzyme - conjugate binding kinetics

The enzyme-conjugate binding kinetics was studied under the optimal conditions for each system by incubating peroxidase - labelled avidin or anti-biotin antibodies for various times (from 5 to 120 minutes). Binding saturation was obtained after 15 minutes of incubation with anti-biotin antibodies whereas 60% binding was observed for

the same incubation time with avidin. Optimal binding was reached after about 100 minutes (Fig. 3). However, the non specific adsorption was higher with peroxidase anti-biotin antibody conjugate as compared to peroxidase-avidin conjugate. The background value was 0.5 unit of optical density for the former conjugate and 0.035 unit for the latter (1 h incubation with conjugate).

DISCUSSION

Pertussis Toxin is an important antigen with multiple pharmacological activities (2, 4). Studies on PT have been hindered by the lack of a rapid and reliable *in vitro* assay (see Introduction). In this work we described and compared different enzyme immunoassays using either microtitration plates or polystyrene beads as solid supports and either peroxidase or biotin-labelled anti-PT IgG conjugates. When biotinylated anti-PT IgG was employed, peroxidase-labelled avidin or peroxidase-labelled anti-biotin antibodies were used to determine anti-PT antibodies specifically bound to PT.

PT can be attached to a polystyrene microplate and detected directly by peroxidase-labelled anti-PT IgG but

this direct ELISA method was moderately sensitive. The detection limit was 4.5 ng of PT. Moreover, the presence of contaminating Bordetella pertussis proteins may compete with PT coating of polystyrene surface. It was therefore better to use a sandwich technique by coating the polystyrene surface with IgG antibodies separated from immunue sera of rabbits immunized with a highly purified toxin preparation. Two procedures were devised using either microtitration plates or polystyrene balls. The comparison of the results obtained for the solid phases clearly showed that the best sensitivity was observed with the plates (Table 1). Indeed, anti-PT antibodies adsorbed on microplates allowed the detection of 0.7 ng whereas the detection limit was 2 ng with the ball-coated antibodies.

Many groups used as we did one step glutaraldehyde method for labelling IgG. This procedure has been successfully employed in various quantitative enzyme immunoassays (33,34). As shown by previous workers, peroxidase activity after coupling retained 70% of the initial activity of native enzyme (35,36). The residual antibody activity in peroxidase labelled anti-PT conjugate was difficult to quantify by conventional immunochemical methods because of the high molecular weight and molecular heterogeneity of enzyme protein conjugates as inferred from

gel filtration and ultracentrifugation analysis. In contrast, no polymerization took place when antibodies were substituted by biotin residues. Moreover, it has been demonstrated that antigen-binding capacity of even heavily biotin-substituted antibody was not significantly affected (27). In this work, we showed that the detection limit decreased 7-fold when biotin-labelled anti-PT IgG were used instead of peroxidase-labelled IgG. Indeed, the detection limit was 0.1 ng PT per sample (0.1 ml) by amplification of the reaction with peroxidase labelled anti-biotin antibodies or avidin.

Avidin is known to adsorb non specifically on various substances and solid phases due to its high iso-electric point (pI ≈ 10) (37,38). We did not observe this non specific adsorption, probably because the peroxidase-avidin conjugate was prepared by using the one-step glutaraldehyde method. Glutaraldehyde reacts with primary amino groups and thus after glutaraldehyde treatment, the pI of avidin is decreased (27). Moreover, the background was much higher when we used peroxidase anti-biotin antibodies conjugate instead of peroxidase-avidin conjugate. This observation can be explained as follows: in order to obtain the same sensitivity and the same detection limit, it was necessary

to dilute enzyme antibody conjugate to 20 mg peroxidase/l and to dilute enzyme - avidin conjugate to 2 mg peroxidase/l. As peroxidase concentration was 10-fold higher with the former conjugate, the consequence was a higher non specific adsorption of peroxidase on antibody coated surface.

It may be thought that using avidin ($K_A \approx 10^{15}$ l/mol) instead of anti-biotin antibodies ($K_A \approx 10^6$ l/mol) should increase the sensitivity of the enzyme immunoassay. In fact, we found that peroxidase-labelled anti-biotin antibodies were as efficient as peroxidase-avidin conjugate for the quantitative assay of PT. The dose response curves obtained with both conjugates were quite superimposable. Moreover, the kinetic study showed that the plateau was reached earlier with peroxidase-antibody conjugate than with peroxidase avidin conjugate. The lack of increase of sensitivity by replacing anti-biotin antibodies by avidin can be explained by the fact that avidin has a high affinity for free biotin and probably a lesser affinity for biotin covalently linked to a protein. On the other hand anti-biotin antibodies were obtained by rabbit immunizations with biotinylated-BSA and probably the

affinity measured with free biotin did not reflect the true affinity of anti-biotin antibodies. Thus association constant of anti-biotin antibodies and avidin for free biotin could not be compared when developing ELISA to select either reagent. Moreover, the background differed from one biological system to another. In conclusion, the most efficient ELISA system for the quantitative determination of pertussis toxin among those tested was anti-PT IgG coated microplate, biotin-labelled anti-PT IgG and peroxidase-avidin conjugate. This enzyme immunoassay allows the detection of as low as 0.1 ng in 0.1 ml samples. It might be particularly suitable for toxin monitoring during purification processes. It is to be noted that a better sensitivity allowing the detection of 50 - 100 pg of toxin per ml can be obtained by the in vitro microplate assay based on the clustering effect of PT on chinese hamster ovary cells (19). However this technique is less convenient as compared to the method described here for routine and less expensive assays.

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