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Evaluation of Whole Cell Vaccine-induced humoral antibody responses in the Pertussis Serological Potency Test in relation to the Mouse Protection Test

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TABLE OF CONTENTS

ABSTRACT 6

SAMENVATTING 7

1. INTRODUCTION 8

2. LABORATORY ANIMALS, MATERIALS AND METHODS 9

2.1. Laboratory animals 9

2.2. Materials 9
2.2.1. Vaccines 9
2.2.2. *B. pertussis* antigens 9
2.2.3. Reference sera 9

2.3. Methods 10
2.3.1. 18323-Whole Cell ELISA 10
2.3.2. Antigen-specific antibody ELISA's 10
2.3.3. PT neutralisation by pertussis antibodies in culture of CHO-cells 11
2.3.4. Complement Activation of pertussis antibodies in ELISA (18323-CAE) 11
2.3.5. Bactericidal Activity of pertussis antibodies (18323-BAA) 11
2.3.6. Influence of the i.c. challenge on antibody responses during the MPT 12
2.3.7. Pertussis Serological Potency Test 12
2.3.8. Intracerebral Mouse Protection Test 12
2.3.9. Comparative study of PSPT and MPT 13

3. RESULTS 14

3.1. Influence of the i.c. challenge on antibody responses during the MPT. 14

3.2. Evaluation of antigen-specific antibody responses in the PSPT. 14

3.3. Evaluation of the functionality of pertussis antibodies in the PSPT. 15

4. DISCUSSION 16

5. CONCLUSIONS 19

REFERENCES 20

TABLES 23

Table 1. Influence of the i.c. challenge on the antibody responses during the MPT 23

Table 2. Correlation between the concentration of pertussis antibodies and antibody mediated complement activation during the MPT. 24

Table 3. Homogeneity of the MPT and PSPT in the χ^2-test. 24

Table 4. Antigen specific antibody responses in the PSPT. 25

Table 5. Correlation between concentration and complement mediated activity of pertussis- or antigen-specific (subclass) antibodies in in vitro assay's. 26

Table 6. Homogeneity of the MPT and PSPT based on complement mediate pertussis antibody responses in χ^2-test. 26

FIGURES 27

Figure 1. Correlation between MPT and PSPT based on pertussis or antigen specific antibodies. 27

Figure 2. Correlation between concentration and complement mediated activity of pertussis antibodies in in vitro assay's. 28

Figure 3. Correlation of MPT and PSPT based on complement mediated pertussis antibody responses. 29

ABSTRACT

The Pertussis Serological Potency Test (PSPT) has been developed as an alternative for the current MPT. The PSPT is based on *in vitro* assessment of the humoral immune response against the whole range of surface-antigens of *B. pertussis* in mice after immunization with Whole Cell Vaccine (WCV). The concentration of pertussis antibodies in sera is measured in the 18323-Whole Cell ELISA (18323-WCE). In an in-house validation study 13 WVC's were tested in the PSPT and the MPT. Homogeneity of both test systems was proven by means of a modified chi-square test; potencies were not significantly different ($p = 0.95$). Compared to the MPT, the PSPT is more reproducible as is indicated by its smaller 95% confidence intervals.

Additionally, the immunogenicity of WVC's has been studied in antigen specific ELISA's and *in vitro* functional test systems to assess correlation with mouse protection. Estimation of WCV-potencies based on the antibody concentration against Pertussis Toxin (PT), Filamentous-Hemagglutinin (FHA) or 69-kDa Outer Membrane Protein (OMP) was not possible due to very low antibody responses which were not vaccine dose dependent. The anti-92-kDa OMP antibody response showed a poor correlation with the MPT, due to scattering. WVC's hardly induced PT-neutralizing antibodies, therefore we have focused on antibody-dependent complement mediated immunity. Although, the concentration of pertussis antibodies (18323-WCE) corresponded well with the complement activating capacity ($R = 0.845$), and the bactericidal capacity ($R = 0.821$) of the analyzed sera, the estimated potencies based on the two test systems showed a poor correlation with the MPT. In conclusion, the protection of mice against a lethal intracerebral challenge is not related to a humoral immune response against a single 'protective' antigen, nor restricted to a single immune mechanism, but may be related to a synergistic effect of humoral responses against a wide range of 'protective' and 'non-protective' antigens. The PSPT is therefore a good alternative for the MPT and provides more precise information about immunogenicity, potency and hence on consistency in production of pertussis WVC's.

SAMENVATTING

De Pertussis Serologische Potency Test (PSPT) is ontwikkeld als een alternatief voor de huidige Muisbeschermingstest (MBT). De PSPT is gebaseerd op het *in vitro* bepalen van de humorale afweerrespons tegen het hele scala aan oppervlakte antigenen van *B.pertussis* bacterie in muizen na immunisatie met kinkhoest whole cell vaccins (WCV). Kinkhoest-antilichaamconcentraties in muizensera worden bepaald met behulp van de 18323-whole cell ELISA (18323-WCE). Tijdens een "in-house" validatie studie zijn 13 kinkhoest WVC's in zowel de PSPT als de MBT getest. De overeenkomst van beide testen is aangetoond m.b.v. een chi-kwadraat test; de werkzaamheid van WVC's in beide testen zijn niet significant verschillend ($p = 0.95$). Vergeleken met de MBT is de PSPT beter reproduceerbaar, hetgeen tot uiting komt in de kleinere 95% betrouwbaarheidsintervallen.

Aanvullend hierop is de immunogeniteit van WVC's in antigeen-specifieke ELISA's en *in vitro* functionele testsystemen bestudeerd. De schatting van WCV-werkzaamheid op basis van de antilichaam responsen tegen Pertussis Toxine (PT), Filamentous Heamagglutinin (FHA) of 69-kDa Outer Membrane Protein (OMP) was niet mogelijk doordat deze te laag en niet dosisafhankelijk waren. De antilichaam respons tegen het 92-kDa OMP correleerde niet met de overleving van muizen in de MBT, door te grote fluctuaties. Omdat WVC's nauwelijks aantoonbare PT-neutraliserende antilichamen opwekken, hebben wij ons toegelegd op het antilichaam afhankelijke en complement gemedieerde afweermechanisme. Hoewel de concentratie kinkhoest antilichamen in de geteste sera goed correleert met zowel het vermogen van deze antilichamen om het klassieke complementsysteem te activeren ($R = 0.845$) en de bactericide "killing" te bevorderen ($R = 0.821$), kwam de werkzaamheid gebaseerd op de concentratie functionele antilichamen in beide testen niet overeen met de werkzaamheid uit de MBT.

De bescherming van muizen tegen een letale intracerebrale challenge is niet gerelateerd aan de humorale antilichaam respons tegen één specifiek antigeen, noch beperkt tot één afweermechanisme, maar lijkt overeen te komen met een synergistische effect van de humorale afweer respons tegen diverse zogenaamde 'beschermende' en 'niet-beschermende' antigenen. De PSPT is daarom een goed alternatief voor de MBT en geeft bovendien meer en betere informatie over de immunogeniteit, werkzaamheid en consistentie in productie van kinkhoest WVC's.

1. INTRODUCTION

The only potency assay for pertussis vaccines that has shown correlation with protection in children is the intracerebral (i.c.) MPT.[2] However, there is considerable dissatisfaction as the MPT has a significant intra- and interlaboratory variation,[3] requires large numbers of animals and a lethal challenge procedure. Therefore we have developed the PSPT[4] as an alternative for the MPT with the ultimate goal of refining and reducing the use of animals. The PSPT is based on the *in vitro* assessment of the humoral response against a wide range of surface-antigens of *B. pertussis* in mice after immunisation with WCV. Mice are immunized intraperitoneally (i.p.) with graded doses of vaccine and bled after four weeks. Sera are titrated in an Enzyme-Linked Immunosorbent Assay (ELISA) with *B. pertussis* whole cells strain 18323 as coating. Potency, based on vaccine dose-dependent antibody responses, is estimated by means of parallel line analysis. Although Dellepiane et al.[5] showed a correlation between protection in the MPT and anti-PT antibodies at the day of challenge, others were unable to detect anti-PT antibodies after immunization with WCV,[6,7] or did not find correlation between mouse protection and antigen-specific,[8,9] or biological activities of WCV-induced pertussis antibodies.[10]

We have demonstrated - in an in-house validation study with thirteen batches of eight different manufacturers - that the potencies of WVC's estimated in the PSPT and MPT were similar in a chi-square (χ^2) test of homogeneity.[4] Additionally, we have evaluated the individual serum samples from this in-house validation study in ELISA for antibody levels against four 'protective' *B. pertussis* antigens; PT, FHA, 69-, and 92-kDa OMP. When possible, potencies based on the antigen-specific antibody responses were estimated and correlated with the MPT.

Measurement of pertussis antibody levels is more meaningful if there is functional relevance, i.e. determination of the ability of antibodies to inhibit virulence associated effects of antigen or organism. We have focused on the relation between the concentration and the biological activity of pertussis antibodies in *in-vitro* assays. PT-neutralising antibodies were measured in a Chinese Hamster Ovary (CHO)-neutralisation assay. Activation of the classic complement route by pertussis antibodies was measured in a Complement Activation ELISA (CAE), and bactericidal killing in a Bactericidal Antibody Assay (BAA).

2. LABORATORY ANIMALS, MATERIALS AND METHODS

2.1. Laboratory animals

N:NIH/RIVM outbred mice were bred and housed under specific pathogen-free conditions at the Central Laboratory Animal facility of our institute. For both tests, PSPT and MPT, equal numbers of both sexes were used according to the specifications as given in the next paragraphs.

2.2. Materials

2.2.1. Vaccines

The lyophilised *B. pertussis* whole cell in-house reference Kh 85/1 has a potency of 30 International Units (IU) per ampoule and contains 40 Opacity Units per millilitre (OU/ml) after reconstitution in 5 ml Phosphate Buffered Saline (PBS). Diphtheria-Pertussis-Tetanus-polio (DTP-polio) vaccines used in this study were routinely produced in our institute and contain 16 OU/ml. DTP vaccines were supplied by seven other manufacturers and contain 32-40 OU/ml. JNIH 3 is an a-cellular vaccine obtained from the Japanese National Institute of Health, which contains PT and FHA.

2.2.2. *B. pertussis* antigens

Purified FHA, 69-kDa OMP and genetically detoxified PT-9K/129G were kindly provided by R. Rappuoli (Sclavo, Italy). Purified 92-kDa OMP was isolated in our institute (manuscript in preparation). Whole Cell Suspension (WCs) of *B. pertussis* strain 18323 (50 OU/ml) was a heat-inactivated 24 hour's culture in PBS, grown on Bordet-Gengou (BG) plates (SVM, The Netherlands).

2.2.3. Reference sera

A pooled Hyper Immune Serum 4 (HIS 4) was used as reference serum to measure antibodies against 18323-whole cells (WC), 69-, and 92-kDa Outer Membrane Protein (OMP) in ELISA. Mice were immunized i.p. at day 0 and 14 with 62.5 µl reference vaccine Kh 85/1, bled at day 28. Sera were pooled and inactivated at 56°C.

HIS 7 was the reference for anti-PT, and anti-FHA antibodies. It was produced by immunizing mice subcutaneously (s.c.) at day 0 and 28 with JNIH 3. Mice were bled at day 42 and sera pooled and inactivated.

2.3. Methods

2.3.1. 18323-Whole Cell ELISA

The 18323-WCE was carried out as follows[4]; Polyvinyl chloride (PVC) micro-plates were evaporated (Titertek, The Netherlands) with 18323-WC suspension (0.8 OU/ml) per well. Non specific-binding sites were blocked with 0.5 % bovine serum albumin (BSA; Organon Technica, The Netherlands) in PBS. Serum samples were analyzed in fivefold serial dilution's of 4 dilution's starting at 1/1000 in diluent (0.5% BSA and 0.05% Tween 80 (Merck, Germany) in PBS). Reference serum HIS 4 was serially diluted in two separate threefold serial dilution of 8 dilution's starting at 1/5000. IgG antibody concentrations were assessed by using the biotin streptavidin-peroxidase system (Amersham, UK); one incubation with goat anti mouse IgG-biotin followed by an incubation with streptavidin-peroxidase. For assessment of IgG subclass antibodies anti IgG_1-, IgG_{2a}-, IgG_{2b}-, or IgG_3-biotin (Amersham, UK) was used. Finally, binding was visualized by addition of tetramethylbenzidin (Sigma, USA) substrate solution. The colouring reaction was stopped by adding 2 M sulphuric acid. Absorbance was measured and the antibody concentrations were calculated by means of a four-parameter fitting analysis and expressed as ELISA Units per millilitre (EU/ml). HIS 4 was assigned to have an antibody concentration of 5000, 2000, 1000, 1000 and 1000 EU/ml for IgG_{tot}, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3, respectively.

2.3.2. Antigen-specific antibody ELISA's

The ELISA for detection of anti-PT antibodies was described by Sato *et al.*[11] Briefly, 1 microgram (mg) per well-purified PT-9K/129G in PBS was added to fetuine (Sigma, USA) pre-coated PVC-micro plates (Titertek, The Netherlands), followed by blocking non-specific binding sites and serial dilution's of serum samples and reference serum. IgG titres were assessed by using goat anti mouse IgG-peroxidase (Cappel, Belgium). Visualization and calculations were performed as described above.

Specific anti-FHA, 69- and 92-kDa OMP antibodies were measured as follows; approximately 1 mg/well purified antigen in 0.1 M carbonate buffer pH 9.0 was coated into PVC-micro plates (Titertek, The Netherlands) and further performed as described above. HIS 4 was also used as reference serum in anti 69-, and 92-kDa OMP antibody ELISA and was assigned to have an antibody concentration of 25, and 1000 EU/ml, respectively. HIS 7 was used as reference for measurement of anti-PT, and anti-FHA antibodies in ELISA and was assigned to have an antibody concentration against both antigens of 1000 EU/ml.

2.3.3. PT neutralisation by pertussis antibodies in culture of CHO-cells

The method used to evaluate the level of PT-neutralizing antibodies induced by vaccination was essentially the same as described by Gillenius *et al.*[13] Neutralizing titres were expressed as the reciprocal of the highest serum dilution causing complete inhibition of the clustering activity of active PT on CHO cells.

2.3.4. Complement Activation of pertussis antibodies in ELISA (18323-CAE)

The capacity of pertussis antibodies to activate the complement system of the classical pathway was determined by measuring Complement factor 3 (C_3) -deposition on *B. pertussis* 18323-WC in an ELISA (CAE). PVC micro-plates with 0.12 OU/ml 18323-WC suspension in PBS were evaporated overnight at 37°C. Non-specific binding sites were blocked with 1% BSA in PBS for 30 min. at 37°C. Reference serum HIS 4 and pooled serum samples were titrated in a two-fold serial dilution range in diluent (PBS, 0.5% BSA, 0.05% Tween 80) and incubated for 1 hour at 37°C. Followed by an incubation of 1 hour with 0.1% fresh guinea-pig (gp) serum as source of complement, diluted in GVB^{4+}-Tw (isotonic veronal buffered saline containing 0.1% gelatine (SVM, The Netherlands), 0.15 mM $CaCl_2$ (Merck, Germany), 0.5 mM $MgCl_2$ (Merck, Germany) and 0.1% Tween 80). C_3-depositions were assessed by using goat anti-gp C_3-peroxidase (Cappel, Belgium) in diluent, for 1 hour at 37°C. Finally, binding was visualized by addition of 100 ml/well TMB-substrate solution. The peroxidase-substrate reaction was stopped after 10 minutes at room temperature with 50 µl/well 2M H_2SO_4. The complement activating capacity of pertussis antibodies was expressed as Complement Activating Units per ml (CAU/ml). HIS 4 was assigned to have a complement activity of 5000 CAU/ml.

2.3.5. Bactericidal Activity of pertussis antibodies (18323-BAA)

Bactericidal antibody levels were determined by a modified method as was described by Poolman *et al.*[14] Two-fold serial dilution's of reference serum HIS 4 and pooled serum samples were prepared in 1% casamino acid solution (SVM, The Netherlands). As source of complement 2% fresh guinea-pig serum in PBS was used. The test organism was a 24-hour culture of *B. pertussis* strain 18323, grown on BG-plates and identical to the challenge strain in the MPT. Serum dilution's (50 µl) were pre-incubated in polystyrene round-bottom micro plates (Greiner, The Netherlands) for 30 min at 35°C with 25 µl of virulent 18323 (5.10^6 cfu/ml) in casamino acid, after which 2% fresh guinea pig complement (25 µl) was added to the reaction-mixture and incubated for 1 hour at 35°C. Five µl mixture of each well was transferred to square BG-plates and the drop was allowed to evaporate. BG-plates

were incubated for three days at 35°C. Positive and negative controls were included and the end-point was taken microscopically as the serum dilution killing 50% of the bacteria. The bactericidal activity was expressed in Bactericidal Units per ml (BU/ml) by the following formula: titre sample/titre reference x 1000.

2.3.6. Influence of the i.c. challenge on antibody responses during the MPT

The antigen-specificity and biological activity of pertussis antibodies induced by RIVM produced WVC's were determined. Briefly, mice (10-14 g) were immunized i.p. at day 0 with a protective dose of the in-house reference vaccine Kh 85/1 (62.5 µl) or DTP-polio vaccine (100 µl). Blood samples were taken at day 17, 20, 24 and 28. Half of the groups of immunized mice were challenged i.c. at day 14, while the other half remained untreated. Sera were obtained and pooled in equal volumes per group of mice. The concentrations of pertussis antibodies in the pooled sera were measured in the 18323-WCE, anti-PT and FHA ELISA's (n = 28), the biological activity in the CHO-neutralization assay, 18323-CAE and 18323-BAA.

2.3.7. Pertussis Serological Potency Test

The PSPT was performed as described recently.[4] Briefly, NIH outbred mice of 20-24 grams in weight were immunised (i.p) with a two-fold dilution range of reference vaccine Kh 85/1 and WVC's under test. For DPT-polio vaccine doses of 80, 40, 20 and 10 µl were used, for the other DPT vaccines 50, 25, 12.5 and 12.5 µl. Twelve mice per vaccine-dose were used. After a four week interval mice were bled and sera were titrated individually in the 18323-WCE. The individual antibody concentrations per vaccine dose were used to estimate the potency by means of parallel line analysis.

2.3.8. Intracerebral Mouse Protection Test

The MPT was performed according to WHO guidelines.[12] NIH outbred mice of 10-14 grams in weight were immunized (i.p.) with a five-fold dilution range of reference and tested vaccines. Sixteen mice per vaccine dose were used. After fourteen day's mice were challenged i.c. with virulent *B. pertussis* of strain 18323. The number of dead mice was recorded daily until day 28. For calculation, only mice dying from day 17 to 28 were taken in account. Based on the percentage of surviving mice per vaccine dilution, the potency of vaccines is estimated by means of probit analysis.

2.3.9. Comparative study of PSPT and MPT

Thirteen pertussis WVC's were tested in the PSPT and MPT, four DPT-polio and nine DPT vaccines. Sera of the PSPT's were additionally analyzed for specific antibodies against PT, FHA, 69- and 92-kDa OMP's and for functional antibodies in CHO-neutralisation assay, 18323-CAE and 18323-BAA. Similarity between the corresponding potencies determined in the MPT and PSPT was estimated by means of a modified chi-square (χ^2)-test of homogeneity.[15] The ratios of both estimates, in which the variances of individual potencies of a test are used as a weighing coefficient, were calculated and used in the χ^2-test of homogeneity. Both test systems are similar if the ratio does not differ significantly from 1.00 ($p > 0.05$). Correlation between antibody concentrations and biological activity of sera was estimated by means of regression analysis.

3. RESULTS

3.1. Influence of the i.c. challenge on antibody responses during the MPT

The antigen-specificity and biological activity of pertussis antibodies induced by RIVM produced WVC's were determined. The i.c. challenge had a clear booster effect on antibody responses against whole cells, and probably for PT too (Table 1), but not on antibody response against FHA. All sera were negative for PT-neutralizing antibodies in the CHO-neutralization assay, but capable of activating the complement system in the 18323-CAE and 18323-BAA (Table 1). As shown in Table 2, the concentration of pertussis antibodies assessed in 18323-WCE correlated well with the capacity of the analysed sera to activate the complement system in the 18323-CAE and to a lesser degree with their capacity to enhance the complement-mediated killing of *B. pertussis* in the 18323-BAA. The i.c. challenge enhanced the bactericidal capacity of pertussis antibodies but not their capacity to activate the complement system.

3.2. Evaluation of antigen-specific antibody responses in the PSPT

In an in-house validation study thirteen different batches of WCV, prepared in different ways and of widely differing potencies from eight manufacturers, were tested in the MPT and the PSPT. Potencies estimated in the PSPT - based on the antibody response against 18323-WC - correlated well with the corresponding potencies estimated in the MPT (Fig. 1a). Both tests were similar with respect to homogeneity according to the χ^2-test (Table 3). Additionally, we have tested the sera also for antibodies against PT, FHA, 69- and 92-kDa OMP in ELISA. In general, the reciprocal antibody-titre's against *B. pertussis* WC, 92-kDa OMP, 69-kDa OMP, FHA and PT were in proportion of 100 to 10 to 1 to < 1 to < 1, respectively. Hyper- and non-responders were mainly found in antibody responses against PT, FHA and to a lesser degree against 69-kDa OMP. In Table 4, the antibody responses are expressed as the mean antibody concentration per vaccine dose (EU/ml), after log transformation to obtain a normal distribution. Nearly all WVC's induced vaccine dose-dependent antibody responses against *B. pertussis* 18323-WC, 92- and 69-kDa OMP's and some against FHA. Only vaccine D induced a significant antibody response against PT. Considering the potency, WVC's differed widely in their proportions of antibodies against 92-, 69-kDa OMP and FHA, some characteristic for the production method of the manufacturers. Furthermore, it is noticeable that the in-house reference Kh 85/1, which is protective

in the MPT, induced very low antibody levels against PT, FHA and 69-kDa OMP. Consequently, the potency of the tested vaccines based on antigen-specific antibody responses could not be estimated, or in case of potencies based on the antibody response against 92-kDa OMP were often statistically invalid - ED_{50} standard deviations were too large - and did not correlate with the potencies obtained by the MPT (Fig. 1b). The corresponding potencies were significantly different in the χ^2-test ($p < 0.001$), as is shown in Table 3.

3.3. Evaluation of the functionality of pertussis antibodies in the PSPT

Sera from PSPT in-house validation study were pooled in equal volumes per vaccine dose (n = 78). Besides the concentration of IgG-antibodies against 18323-WC, FHA, PT, 92- and 69-kDa OMP's, IgG subclasses against 18323-WCs and 92-kDa OMP were also measured. The antibody concentrations were correlated to the PT-neutralizing, complement activating and bactericidal capacity of the analysed sera. The proportion of the reciprocal antibody titre's of the pooled sera were essentially the same as the individually tested sera; 18323-WC >> 92 kDa OMP >> 69 kDa OMP > FHA > PT. The biological antibody activity was assessed in the CHO-neutralization assay, 18323-CAE and 18323-BAA. Only one of the pooled sera induced a measurable amount of PT-neutralizing antibodies. The capacity of the sera to activate the classical pathway of the complement system (18323-CAE) and/or to induce bactericidal killing of virulent *B. pertussis* (18323-BAA) is related to the concentration of antibodies against the whole wide range of *B. pertussis* surface antigens, R = 0.845 and 0.821 respectively (Fig.2A and 2B). As is shown in Table 5, complement activation and bactericidal killing may depend on antigen- and IgG subclass-specificity of the antibodies. Anti 92-kDa OMP antibodies may be involved in complement activation and bactericidal killing, antibodies against 69-kDa OMP and PT apparently not. The best correlation's between the concentration and biological activity of pertussis antibodies were found with IgG_{2b}- and IgG_3 anti 18323-WC subclass antibodies, and IgG_{2a}- and IgG_3 anti 92-kDa OMP subclass antibodies. Based on the limited results of the 18323-CAE and -BAA potencies were estimated and did not correlate with protection in the MPT, as is shown in Figure 3 and Table 6.

4. DISCUSSION

Recently we have demonstrated that the survival of mice in the MPT is related to the concentration of pertussis antibodies against a wide range of surface-antigens at the day of challenge.[4] We showed that the i.c. challenge has a booster effect on the WCV-induced antibody response in the MPT. This booster effect differed per test and per vaccine, and may contribute to the poor reproducibility of the MPT. In this study we now show that the i.c. challenge not only potentiates, but also modulates antibody responses (Table 1 and 2). The i.c. challenge booster effect differed per antigen and clearly enhanced the capacity to activate the classical pathway of the complement system, but not the bactericidal capacity of the serum pertussis antibodies. The PSPT makes the i.c. challenge superfluous and may therefore be more reproducible.[4]

In an in-house validation study we show that the MPT and the PSPT - based on the antibody response against a wide range of surface-antigens of *B. pertussis* strain 18323 - are similar in a χ^2-test of homogeneity (Table 3). In addition, we have tested the sera in several antigen-specific ELISA's and functional *in vitro* assays to gain more information about immunogenicity of WVC's and consequently about consistency in production. We have chosen to evaluate the antibody responses against four 'protective' *B. pertussis* antigens; PT, FHA, 69- and 92-kDa OMP's. Active immunization with 69-kDa OMP, FHA or PT and passive transfer of polyclonal and/or monoclonal antibodies against these antigens were protective in lethal respiratory or aerosol challenge models.[16] Detoxified PT was also protective in the MPT.[16] 92-kDa OMP was chosen because RIVM vaccines induce mainly antibodies against OMP's and the presence of 92-, 32-, and 30-kDa OMP's greatly enhances the protective capacity of outer membrane vesicles.[14] Sera were determined individually for antigen-specific antibodies and, when possible, potencies were estimated. As shown in Table 4, WVC's of different manufacturers differed in their antigen-specific antibody responses especially against 92- and 69-kDa OMP. In general, all WVC's induced vaccine dose-dependent antibody responses against 92-, and 69-kDa OMP and some against FHA. Just one WCV induced a reliable vaccine dose dependent antibody response against PT. It is noticeable that the antigen-specific antibody responses may be characteristic for the production method of the manufacturer. Considering the individual potency, the RIVM-vaccines J to M (Table 4) seem to differ in their antigen-specific antibody responses

compared with the products of the other manufacturers. The main difference in production is the method of detoxification. RIVM-vaccines are heat-inactivated instead of chemically detoxified and contain hardly detectable amounts of native PT.

WCV potencies based on the concentration of specific antibodies against PT, FHA and 69-kDa OMP could not be estimated due to low antibody responses or in the case of 92-kDa OMP did not correlate with protection in MPT, due to scattering (Table 3). We therefore assume that WCV-induced protection in the MPT is not related to a specific antibody response against one single 'protective' antigen such as described for PT[5], but to a synergistic effect of antibody responses against a wide range of surface-antigens.

The functional activity of WCV-induced antibodies may be of more importance. Pooled sera of the in-house validation study were tested in several *in vitro* assays. WVC's hardly induced anti-PT antibodies and consequently the tested sera were not able to neutralize *in vitro* PT-toxicity, as was shown earlier.[6,7,17] The low amount of PT in WCV[18] is probably insufficient to induce protection by PT-neutralizing antibodies. The biological relevance of anti-adherence antibodies was not investigated, as we assume that antibodies against adhesins may be irrelevant in protection against an i.c. challenge.[16,19] Roberts *et al.*[21] presumed that the effector mechanisms of anti 69-kDa OMP antibodies may be of minor importance in preventing adherence of *B. pertussis* to respiratory tract epithelium and could be involved in complement mediated lysis or opsonization of *B. pertussis*. We therefore focused on complement mediated immune responses against virulent *B. pertussis*. The capacity of the pooled sera to activate the complement classical pathway was tested by measuring the C_3-depositions on 18323-whole cells in ELISA and the bactericidal killing of virulent *B. pertussis* strain 18323. As shown in Figure 2a, all sera were capable of activating the complement system and the amount of C_3-depositions on 18323-WC was in proportion to the concentration of pertussis antibodies (R = 0.845). The capacity of pertussis antibodies to activate the complement system indicates that antibody-dependent cell-mediated cytotoxicity e.g. phagocytosis may be involved in protection against the i.c. challenge. Preliminary results of phagocytosis *in vitro* experiments with FITC-labelled *B. pertussis* in a whole blood system showed that WCV-induced pertussis antibodies enhanced phagocytosis (manuscript in preparation). Correlation between the bactericidal capacity and concentration of pertussis antibodies (R = 0.821) is shown in Figure 2B. Nevertheless, bactericidal

antibodies are regarded as non-protective and correlation between *in vitro* activity of bactericidal antibodies and protection in the MPT to be fortuitous.[10,14] On the other hand, 'protective' antigens such as 69-kDa OMP and FHA administered as a single component vaccine also failed to pass the MPT, but administered in combination with other 'protective' antigens enhanced protection.[22] We assume that bactericidal antibodies may enhance protection against the i.c. challenge.

As could be expected, complement-mediated immunity is not restricted to a single antigen-specific antibody response. Table 5 shows the correlation coefficients between the functional *in vitro* assays and ELISA's for antigen-specific antibodies. Antibodies against 92-kDa OMP may play a role in complement activation, antibodies against 69-kDa OMP and PT apparently not. All WVC's induced the highest antibody responses against WCs and 92-kDa OMP, predominantly IgG_{2b} subclass antibodies against whole cells, and IgG_{2a} against 92-kDa OMP. The role of IgG subclass antibodies is still not clear. On the one hand IgG_{2a} and IgG_{2b} antibodies that have a high affinity for Fc-receptors corresponded well with the opsonic capacity and to a lesser degree with the bactericidal capacity of the analysed sera, on the other hand IgG_3 antibodies showed the same correlation. More investigation is required. The discrepancy in opsonic capacity of pertussis antibodies in the MPT (Table 2) and the PSPT (Table 5) may be caused by the fact that RIVM vaccines induced higher antibody responses against 92-kDa OMP than WVC's from other manufactures. To get an indication whether the biological activity of pertussis antibodies in the 18323-CAE or 18323-BAA may be a good parameters to estimate potencies in the PSPT, we have estimated potencies based on the limited data from both tests. Potencies were statistically invalid and showed a poor correlation with the corresponding potencies of the MPT (Table 6 and Fig.3a and 3b). Furthermore, the 18323-CAE and - BAA are laborious and quite difficult to reproduce, and therefore unsuitable as test methods for potency testing.

5. CONCLUSIONS

Protection against i.c. challenge in the MPT is not related to an antibody response against a single 'protective antigen', nor restricted to a single immune mechanism, but may be related to a synergistic effect of humoral immune responses against a wide range of 'protective' or 'non-protective' antigens. Correlation between the MPT and the PSPT confirms that the mean concentration of pertussis antibodies per vaccine dose is a promising substitute to measure humoral immunity against virulent *B. pertussis*, without the variable effects of an i.c. challenge. Compared to the MPT, the PSPT provides more precise information about immunogenicity, potency and hence on consistency in production of pertussis WVC's.

Furthermore the use of the PSPT also leads to a reduction of animal distress and number of animals in use. Moreover, by simplifying the multiple-dose design to a single-dose assay, after consistency in manufacturing and testing has been proven, the number of animals could be reduced even more. Stability of final products or in-process control during production could be monitored easier and cheaper when less animals are used. Another option is combining *in vitro* serological assay's for potency testing of Tetanus-, Diphtheria- and Pertussis components in one animal model.

Further validation such as an international collaborative study to assess intra- and inter laboratory variation in potency and reproducibility could warrant replacement of the MPT by this serological model.

REFERENCES

1. Medical Research Council.
   Vaccination against whooping-cough: relation between protection in children and results of laboratory tests.
   Br Med J 1956; 2:454-462
2. Kendrick P.L., Eldering G., Dixson M.K. and Misner J.
   Mouse protection tests in the study of pertussis vaccines: a comparative series using intracerebral route of challenge.
   Am J Public Health 1947;37:803-810
3. v Straaten-vd Kappelle I., vd Gun J.W., Marsman F.R., Hendriksen C.F.M. and vd Donk H.J.M.
   Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine.
   RIVM 1995; report nr. 100003001
4. vd Ark A., v Straaten-vd Kappelle I., Akkermans A., Hendriksen C. and vd Donk H.
   Development of Pertussis Serological Potency Test: Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model.
   Biologicals 1994;22:233-242
5. Dellepiane N.I., Manghi M.A., Erikson P.V., di Paola G. and Cangelosi A. Pertussis whole cell vaccine: Relation between intracerebral protection in mice and antibody response to Pertussis Toxin, Filamentous Hemagglutinin and Adenylate Cyclase.
   Zbl Bakt 1992;277:65-73
6. Munoz J.J. and Peacock M.G.
   Role of pertussigen (Pertussis Toxin) on the mouse protective activity of vaccines made from Bordetella species.
   Microbiol Immunol 1989;33:341-355
7. Tiru M., Jäätmaa E., Gillenius P. and Aslelöf P.
   Optimization of immunization schedule to standardize antibody response in mice to pertussis vaccines.
   Develop Biol Stand 1985;61:469-475

8 Wardlaw A.C. and Jakus C.N.
Intracerebral mouse protection test for pertussis vaccine. I. Apparent absence of humoral protective antibody under the usual test conditions.
Can J Microbiol 1968;14:989-997

9. Dolby J.M. and Standfast A.F.B.
The intracerebral infection of mice with *Bordetella pertussis*.
J Hyg Camb 1961;59:205-217

10. Ackers J.P. and Dolby J.M.
The antigen of *Bordetella pertussis* that induces bactericidal antibody and its relationship to protection of mice.
J Gen Microbiol 1972;70:371-382

11. Sato H. and Sato Y.
Bordetella pertussis infection in mice: Correlation of specific antibodies against two antigens, pertussis toxin, and filamentous haemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system.
Infec Immun 1984;46:411- 415

12. WHO Expert Committee on Biological Standardization.
Fortieth Report.
WHO Tech Rep Ser 1990;800:136-138

13. Gillenius P., Jäätmaa E., Aslelöf P., Tiru M. and Granström M.
The standardization of an assay for pertussis toxin and antitoxin in micro plates culture of Chinese hamster ovary cells.
Biol Stand 1985;13:61-66

14. Poolman J.T., Hamstra H.J., Barlow A.K., Kuipers B., Loggen H. and Nagel J.
Outer Membrane Vesicles of *Bordetella pertussis* are protective antigens in the mouse intracerebral challenge model.
In: Proceedings of the sixth international symposium on pertussis.
Manclark CR (ed) 1990. DHHS Publication no (FDA) 90-1164:148-155.

15. Finney D.J.
Chapter 14: The Combination of Estimates in Statistical Methods in Biological Assay.
London 1964; Charles Griffin and Company Limmited.

16. Zealey G., Loosmore S., Yacoob R. and Klein M.
Modern Pertussis Vaccines.
Vaccine Research 1992;1:413-427

17. Wiertz E.J.H.J., Walvoort H.C., v Loveren H., v Straaten-vd Kappelle I., vd Gun J.W. and Kreeftenberg J.G.
A-cellular and whole cell pertussis vaccines protect against the lethal effects of intracerebral challenge by different T-cell dependent humoral routes.
Biologicals 1990;18:173-180.

18. Ibsen P.H., Petersen J.W. and Heron I.
Quantification of pertussis toxin, filamentous haemagglutinin, 69-kDa outer membrane protein, agglutinogens 2 and 3 and lipopolysaccharide in the Danish whole-cell pertussis vaccine.
Vaccine 1993;11:318-322

19. Berenbaum M.C., Ungar J. and Stevens W.K.
Intracranial infection of mice with *Bordetella pertussis*.
J Gen Microbiol 1960;22:313-322

20. Manghi M., Dellepiane N., Erikson P.V., di Paolo G. and Dios F.
Antibody response to pertussis toxin and filamentous haemagglutinin in NIH mice immunised with international standard for pertussis vaccine. Biologicals 1990;118:165-172

21. Roberts M., Tite I.J., Fairweather N.F., Dougan G. and Charles J.G.
Recombinant P.69/pertactin: immunogenicity and protection of mice against *Bordetella pertussis* infection.
Vaccine 1992;10:43-48

22. Novotny P., Chubb A.P., Crownly K. and Charles I.G.
Biologic and Protective properties of 69-kDa outer membrane protein of *Bordetella pertussis*.
FEMS lett 1990;67:285-290

TABLES

Table 1. Influence of the i.c. challenge on the antibody responses during the MPT

assay	day	Kh 85/1 reference		DPT-polio	
		- chal.*	+ chal.†	- chal.*	+ chal.†
18323-WCE‡	17	171	208	251	303
	20	247	438	498	648
	24	475	1043	861	990
	28	455	1117	1111	1466
PT-ELISA‡	17	0	5	4	6
	20	12	4	4	5
	24	4	4	7	16
	28	5	124	7	404
FHA-ELISA‡	17	7	4	32	5
	20	5	17	40	37
	24	6	16	59	66
	28	9	8	78	83
18323-CAE§	17	335	395	680	675
	20	460	1083	1203	1569
	24	783	1918	2043	2451
	28	808	2103	2488	3546
18323-BAA°	17	200	150	100	100
	20	200	200	200	200
	24	400	200	200	400
	28	200	400	400	400
CHO-neutr.	17	0	0	0	0
	20	0	0	0	0
	24	0	0	0	0
	28	0	0	0	0

* Sera of non-challenged mice.
† Sera of challenged mice.
‡ EU/ml
§ CAU/ml
° BU/ml

Table 2. Correlation between the concentration of pertussis antibodies and antibody mediated complement activation during the MPT.

assay	18323-CAE		18323-BAA	
	- chal.*	+ chal.†	- chal.*	+ chal.†
18323-WCE	0.954	0.970	0.985	0.814

* Sera of non-challenged mice. † Sera of challenged mice.

Table 3. Homogeneity of the MPT and PSPT in the χ^2-test.

	18323-WCE	92-kDa OMP-ELISA
χ^2	5.32	28.66
ratio	1.042 (0.831-1.360)	1.523 (1.192-1.946)
p	0.95	< 0.001

Table 4. Antigen specific antibody responses in the PSPT.

test	vaccine	dose	18323-WC	92-kDa OMP	69-kDa OMP	FHA	PT	potency§
1	reference	62.50	494	104.6	7.9	0.4	0.2	
		31.25	246	34.2	1.3	0.3	0.0	6.0
		15.62	118	14.0	0.2	0.1	0.0	
		7.81	49	5.8	0.1	0.2	0.0	
	A*	50.00	1298	74.4	67.7	126.9	4.4	
		25.00	906	48.0	21.7	93.3	1.0	25.8
		12.50	639	29.3	7.7	37.4	1.9	
		6.25	231	4.5	0.3	7.1	0.2	
	B†	50.00	984	157.1	28.6	1.1	2.4	
		25.00	615	91.7	15.9	1.0	2.0	44.2
		12.50	494	68.8	1.7	0.4	1.1	
		6.25	191	23.0	0.5	0.2	0.3	
	C	50.00	1429	96.2	107.5	2.4	0.1	
		25.00	761	68.7	32.3	2.0	0.0	15.4
		12.50	283	32.0	0.8	1.1	0.0	
		6.25	98	4.7	0.0	0.3	0.0	
	D	50.00	877	38.2	32.3	1.1	5.7	
		25.00	290	23.9	24.4	0.8	6.8	11.8
		12.50	177	6.9	3.4	0.2	2.9	
		6.25	129	3.0	6.9	0.1	0.3	
2	reference	50.00	184	31.4	0.4	0.4	0.5	
		25.00	84	37.6	0.3	0.1	0.1	6.0
		12.50	65	16.7	0.1	0.0	0.0	
		6.25	31	6.3	0.1	0.0	0.0	
	E	100.00	185	5.1	3.7	10.9	3.1	
		50.00	85	0.2	0.7	1.5	2.0	4.1
		25.00	61	0.1	0.7	0.3	0.2	
		12.50	22	0.0	0.3	0.1	0.0	
3	reference	50.00	873	352.7	7.8	2.7	1.7	
		25.00	370	97.8	0.5	1.5	0.2	6.0
		12.50	110	73.8	0.8	0.1	0.0	
		6.25	66	22.2	0.1	0.1	0.0	
	F	50.00	1368	307.8	27.4	7.5	0.3	
		25.00	700	440.0	17.9	3.5	0.2	9.7
		12.50	237	140.3	7.9	1.0	0.1	
		6.25	142	36.0	2.0	0.2	0.1	
	G†	50.00	741	82.1	42.3	10.9	1.9	
		25.00	615	69.8	16.4	11.9	2.4	17.1
		12.50	411	37.7	23.8	4.2	0.7	
		6.25	244	39.1	0.5	2.5	0.2	
	H	50.00	629	75.1	12.9	18.3	1.4	
		25.00	447	54.9	7.2	13.5	0.6	5.5
		12.50	212	19.1	1.9	1.3	1.1	
		6.25	144	10.7	0.2	0.9	0.9	
4	reference	50.00	231	99.6	0.8	0.3	0.1	
		25.00	144	54.0	0.0	0.3	0.0	6.0
		12.50	84	22.5	1.0	0.0	0.0	
		6.25	47	20.1	0.8	0.0	0.0	
	I*	50.00	681	369.0	17.3	1.6	2.5	
		25.00	364	141.5	12.1	2.1	1.2	19.9
		12.50	310	174.5	2.6	1.2	0.3	
		6.25	130	64.5	2.1	1.3	0.5	
	J‡	80.00	547	188.7	5.4	0.6	0.0	
		40.00	509	221.3	4.7	0.1	0.0	4.5
		20.00	149	36.1	1.9	0.0	0.0	
		10.00	30	4.0	0.9	0.0	0.0	
	K‡	80.00	706	212.6	7.5	0.1	0.0	
		40.00	550	270.8	5.8	0.0	0.0	6.7
		20.00	149	49.5	1.0	1.8	0.0	
		10.00	70	15.3	0.1	0.1	0.0	
	L‡	80.00	512	191.8	11.4	1.4	0.1	
		40.00	412	268.4	1.1	0.0	0.0	9.2
		20.00	209	71.8	10.6	0.9	0.0	
		10.00	85	21.6	5.8	0.0	0.0	
5	reference	50.00	333	66.6	0.6	1.0	2.4	
		25.00	208	13.1	0.4	0.4	0.6	6.0
		12.50	47	4.7	0.1	0.0	0.5	
		6.25	47	3.2	0.1	0.0	0.1	
	M‡	80.00	371	215.5	0.9	1.0	0.3	
		40.00	612	32.2	0.2	0.9	0.2	10.0
		20.00	113	15.0	0.0	0.1	0.3	
		10.00	84	3.8	0.1	0.1	0.1	

*, † and ‡ WVC's of the same manufacturer § potency estimated by means of MPT (IU).

Table 5. Correlation between concentration and complement mediated activity of pertussis- or antigen-specific (subclass) antibodies in in vitro assay's.

assay	18323-CAE	18323-BAA
18323-WCE:		
IgG_{tot}	0.845	0.821
IgG_{1}	0.476	0.731
IgG_{2a}	0.567	0.698
IgG_{2b}	0.887	0.821
IgG_{3}	0.817	0.749
92-kDa OMP ELISA:		
IgG_{tot}	0.689	0.468
IgG_{1}	0.321	0.358
IgG_{2a}	0.767	0.572
IgG_{2b}	0.450	0.550
IgG_{3}	0.764	0.606
FHA ELISA:	0.527	0.567
PT ELISA:	0.213	0.146
69-kDa OMP ELISA:	0.012	0.085

Table 6. Homogeneity of the MPT and PSPT based on complement mediate pertussis antibody responses in χ^2-test.

χ^2 -test	18323-CAE	18323-BAA
χ^2	287.79	131.07
ratio	0.736	1.392
p*	< 0.001	< 0.0001

* Test systems are similar when the ratio does not significantly differ from 1.00 and the p-value > 0.05.

FIGURES

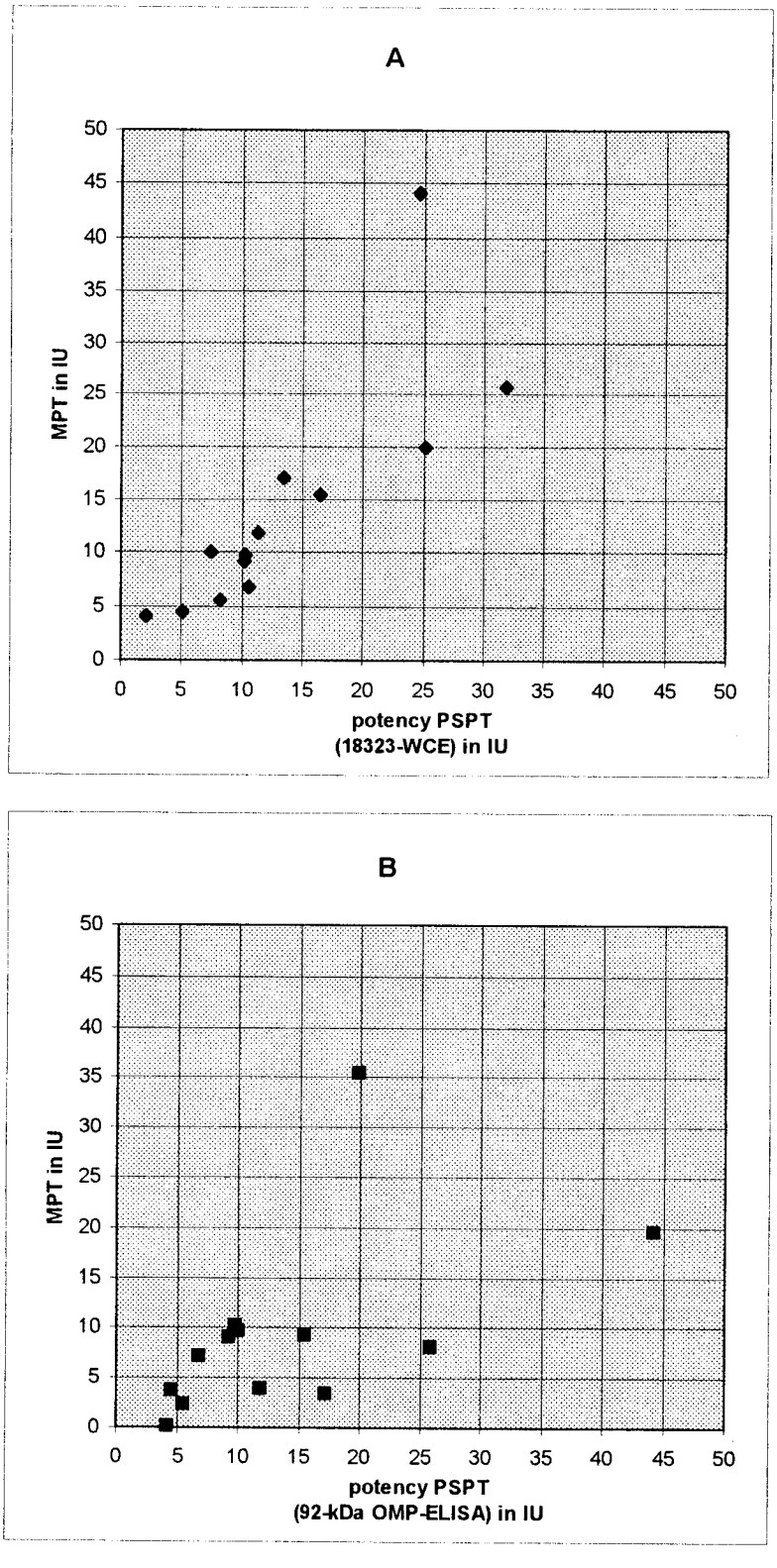
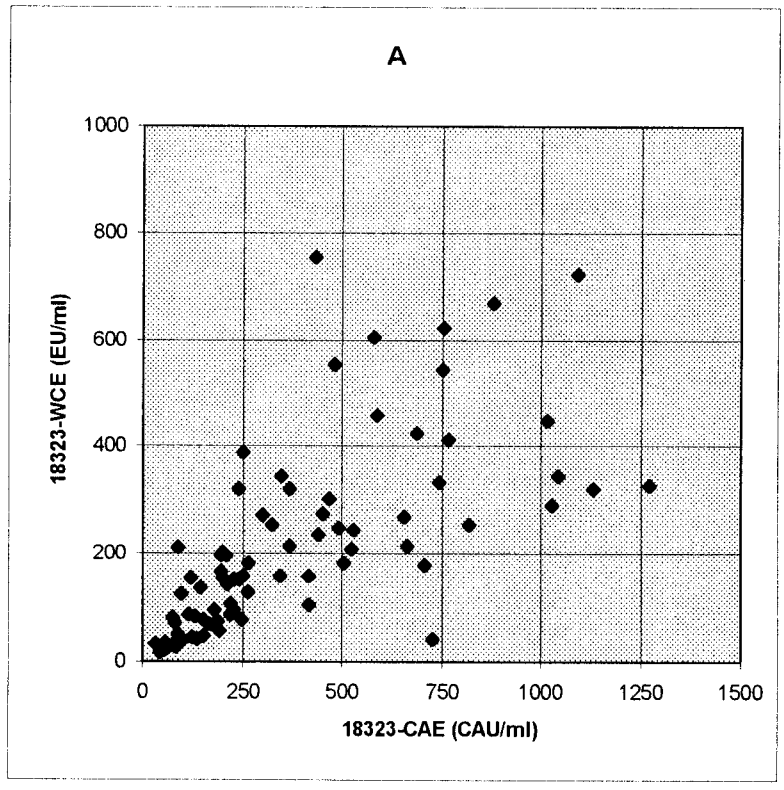


Figure 1. Correlation between MPT and PSPT based on pertussis or antigen specific antibodies.



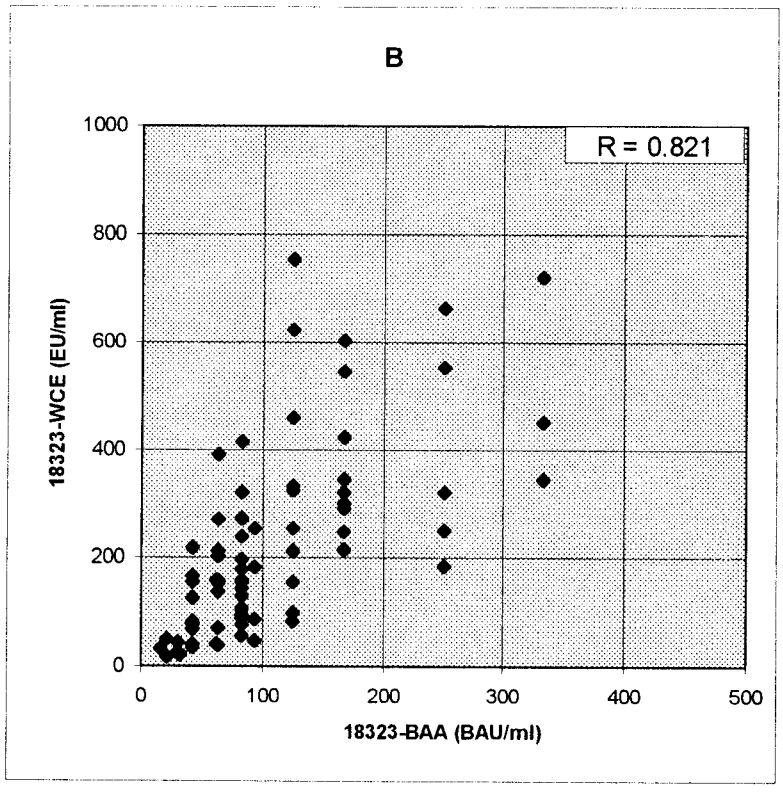


Figure 2. Correlation between concentration and complement mediated activity of pertussis antibodies in in vitro assay's.

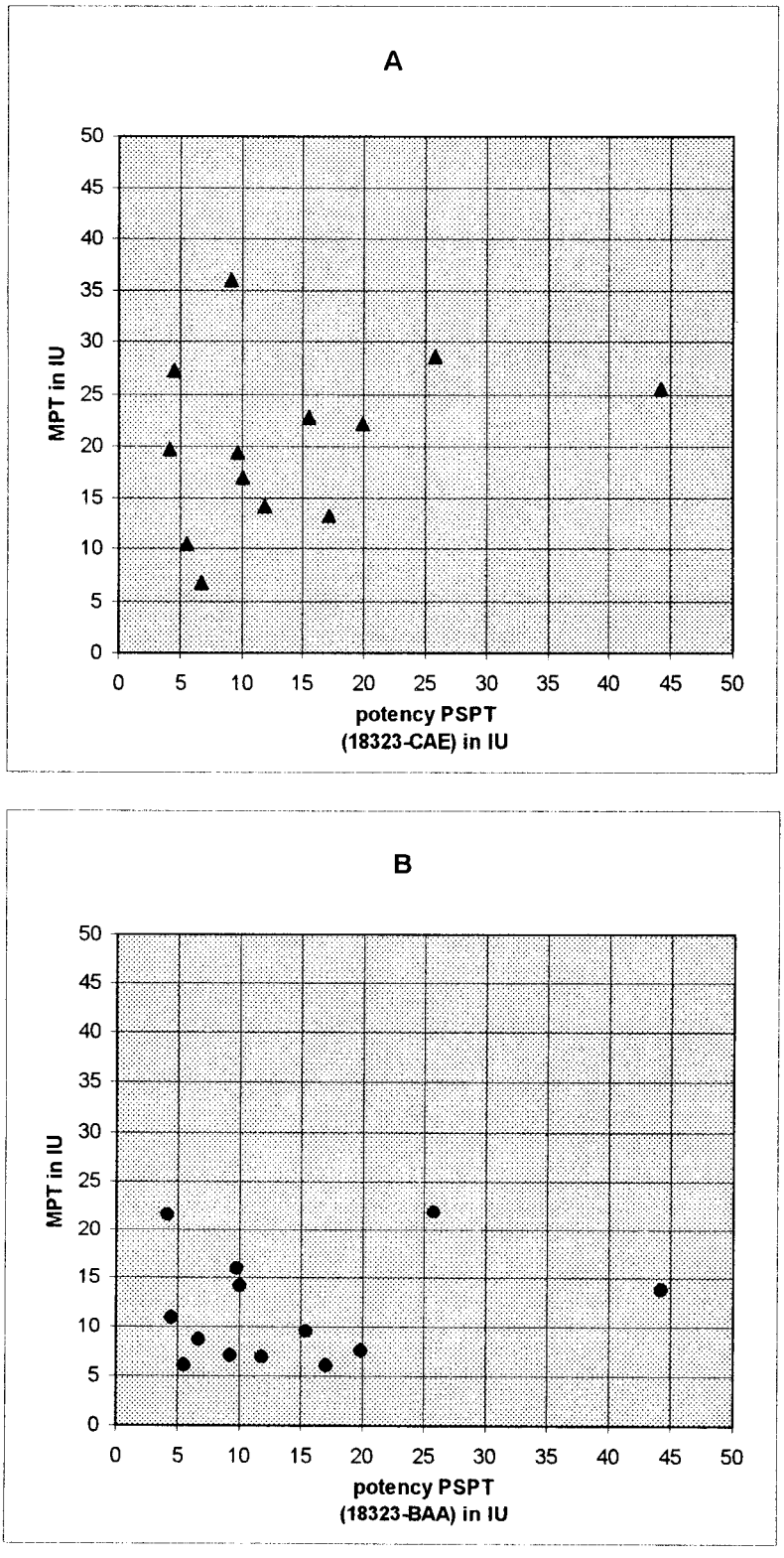


Figure 3. Correlation of MPT and PSPT based on complement mediated pertussis antibody responses.