Enzyme-Linked Immunosorbent Assay-Based Inhibition Test for Neutralizing Antibodies to Polioviruses as an Alternative to the Neutralization Test in Tissue Culture

G. EDEVÅG,¹ B. WAHREN,¹ A. D. M. E. OSTERHAUS,² V.-A. SUNDQVIST,^{1,3} AND M. GRANSTRÖM^{4*}

Department of Virology, Swedish Institute for Infectious Disease Control,¹ Stockholm University School of Health and Science,³ and Department of Clinical Microbiology, Karolinska Hospital,⁴ Stockholm, Sweden, and Institute of Virology, Erasmus University, Rotterdam, The Netherlands²

Received 1 June 1995/Returned for modification 25 July 1995/Accepted 22 August 1995

A poliovirus-binding inhibition test (PoBI test) was established for the quantitative determination of antibodies to polioviruses and was evaluated in comparison with the conventional neutralization test (NT). The first step of the PoBI test is an incubation of serial dilutions of test samples with inactivated poliovirus followed by the detection of free viral epitopes by a double antibody sandwich enzyme-linked immunosorbent assay with type-specific capture polyclonal antisera and type-specific neutralizing monoclonal indicator antibodies. A comparison of the PoBI test with the conventional NT for antibodies to all three types in 100 human serum samples showed excellent correlations ($r > 0.95$) over a wide range of antibody concentrations. The PoBI test, not necessitating live virus and tissue culture facilities, could be a simple alternative to the NT, and the principle of the assay is potentially applicable to other microbial systems.

The amounts of functional antiviral antibodies as determined by neutralization tests (NT) are generally considered to best mirror the level of protection acquired after natural exposure to a disease or after immunization. The drawbacks of NT are, however, numerous: the method is restricted to viruses which either induce visible cytopathogenic effects (CPE) in tissue culture or cause disease in animals. In the latter case, the costs of the assay usually exclude any large-scale testing for neutralizing antibodies. Even in the situation of a reasonably clear-cut CPE, the test requires the use of live virus, a major risk factor in many instances. In all cases, the NT is a cumbersome and labor-intensive method. For these reasons, nonfunctional assays are often used instead of the functional assay. Although a reasonable correlation is usually obtained, the correlation coefficients for low antibody concentrations, which are of major importance in assessing immunity in a population, have generally shown a low degree of correlation between functional and nonfunctional tests (5, 9).

Neutralizing antibodies are also known to best reflect immunity to bacterial toxins, e.g., diphtheria and tetanus toxins. For diphtheria toxin, the *in vivo* rabbit skin test has been replaced by an *in vitro* test with Vero cells, and excellent correlations between results obtained by the two methods have been found (7, 8). The problem of having to use an *in vivo* assay in mice for tetanus toxin, not known to induce CPE in any cell line, has remained unchanged for many years. Nonfunctional assays have been tested but were shown to give overestimates of antibody concentrations in immunized individuals whose actual levels of antibodies were low or undetectable by NT (12). More recently, an *in vitro* tetanus toxin-binding assay using enzyme-linked immunosorbent assay (ELISA) as the detection system has been described, and an excellent correlation between this tetanus toxin-binding test and the *in vivo* NT was shown over a large range of antibody

concentrations (3). While this report was being prepared, an inhibition assay for rabies glycoprotein antibodies was also published and the assay was shown to correlate well with the rapid fluorescent focus inhibition test (4).

The aim of this study was to investigate the possibility of applying the principle of the tetanus toxin inhibition test to detect and quantify antibodies induced by a viral vaccine; poliovirus was chosen as the model system.

MATERIALS AND METHODS

PoBI test. The poliovirus-binding inhibition test (PoBI test) was developed according to the principles of the method for quantifying tetanus antitoxin as described by Hendriksen et al. (3). The test represents a two-step procedure; the first step involves binding the antigen to serial dilutions of the test sample, and the second step involves detecting unbound antigen in the mixture by a sandwich ELISA.

For the first step, wells in round-bottomed polystyrene microtiter plates (model no. 650101; Grainer, Nürtingen, Germany) were blocked with 250 μ l of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline with 0.05% Tween 20 but without Ca and Mg (buffer A) for 1.5 h at 37°C. After blocking, twofold dilutions of the test samples in 75- μ l volumes were made directly in the plates from a dilution of 1:2 to a dilution of 1:2,048. On each plate, an in-house standard was titrated in parallel as a reference. The in-house standard had been calibrated against a World Health Organization standard (the second International Standard serum, no. 66/202, for antipoliiovirus types 1, 2, and 3; World Health Organization, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom) (14, 15) containing antipoliiovirus type 1 (25 IU/ml), antipoliiovirus type 2 (50 IU/ml), and antipoliiovirus type 3 (5 IU/ml). A predetermined concentration of formaldehyde-inactivated trivalent poliovirus vaccine was added to each well in a volume of 75 μ l per well. The vaccine, produced in cynomolgus kidney cells at Rijks Instituut voor de Volksgezondheid (RIVM), contained in lot no. 345 (National Bacteriological Laboratory, Stockholm, Sweden) 20 D-antigen units (DU) of poliovirus type 1 per ml, 4 DU of type 2 per ml, and 16 DU of type 3 per ml (13). Poliovirus type 1 was a Mahoney strain, type 2 was MEF, and type 3 was a Saukett strain. The plates were then incubated for 3 h at 37°C.

In the second step, 100 μ l of the mixture from each well was transferred by a multichannel pipette (Proline electronic; BIOHIT OY, Helsinki, Finland) to flat-bottomed 96-well microplates (Immunolon 2, model no. M 129 B; Dynatech Ltd., Sussex, United Kingdom) previously coated with capture antibodies of polyclonal antisera to the respective poliovirus types. For type 1, an immunoglobulin G fraction of calf antiserum to the Mahoney strain (RIVM) was diluted 1:1,000; for type 2, rabbit whole serum to the MEF strain (National Bacteriological Laboratory) was diluted 1:1,000; and for type 3, calf serum immunoglobulin G to the Saukett strain (RIVM) was diluted 1:1,000. Dilution buffer was 0.05 M sodium carbonate buffer, pH 9.6 (buffer B). After being coated overnight at

* Corresponding author. Mailing address: Department of Clinical Microbiology, Karolinska Hospital, S-171 76 Stockholm, Sweden. Phone: 46-8-729 3564. Fax: 46-8-30 80 99. Electronic mail address: marta@mb.ks.se.

FIG. 1. Example of titration curves obtained by the PoBI test and calculation of antibody concentrations in test samples (filled triangles, sample 1, 0.125 IU/ml; open triangles, sample 2, 4.0 IU/ml) in relation to a standard (filled boxes, reference serum, 1.0 IU/ml). The corresponding dilutions of the test samples and the reference serum are given below the figure. Solid vertical line, value of 1.0 IU/ml obtained from the reference serum; dotted lines, values in international units of samples obtained in relation to the reference serum.

4°C, the plates were blocked with 2% BSA in 100 µl of distilled water for 1 h at room temperature. Thus prepared, the plates could be stored at -20°C for several months prior to use. The ELISA plates with the transferred serum-virus mixtures were incubated overnight at 37°C. Monoclonal indicator antibodies (10, 11) to poliovirus type 1 (Mahoney, monoclonal no. 2-20 F10, 81/27, RIVM) and to poliovirus type 2 (MEF strain no. 5585, 6-15 C6, 81/21, RIVM) were added in a dilution of 1:50,000 in buffer A, and to poliovirus type 3 (Saukett strain no. 27-2-13 A9, 2-13 D9, 81/27, RIVM) they were added in a dilution of 1:10,000 in buffer A, supplemented 1 h before use with 2% inactivated normal serum of the respective species (calf or rabbit) of the capture antibodies to avoid cross-reactions. Incubations with the indicator antibodies were for 2.5 h at 37°C. Rabbit anti-mouse immunoglobulin G, conjugated with alkaline phosphatase (Sigma Chemical, St. Louis, Mo.), was diluted 1:500 in buffer A supplemented with 2% normal calf serum to avoid cross-reactions with capture type 1 and 3 antibodies. Conjugate incubations were for 2 h at 37°C. In the ELISA, 100-µl volumes were used throughout the procedure and all washings were made four times in buffer A without BSA. Incubations with the substrate, *p*-nitrophenylphosphate, were at room temperature until the highest values of the in-house standard reached a value of approximately 1.0 at A_{405} . The first serum dilution with an absorbance increase of 0.1 nm (above an A_{405} of 0.1) was taken as the titer of the test sample. The titer was expressed in international units per milliliter against the calibrated in-house standard (Fig. 1).

The specificities of the monoclonal antibodies were tested by NT, and a neutralization of type 2 poliovirus was seen at a dilution of 1:10 with the type 1 and 3 antisera. No other cross-reactions were found. In the PoBI test, however, these monoclonal antibodies were used at dilutions of 5×10^{-4} to 10^{-5} (see above). The homologous neutralizing titer of the monoclonal antibodies was 10^{-6} for type 1 and 10^{-9} for types 2 and 3.

NT. The serum samples were titrated in 96-well microtiter plates (Nunc, Delta, Nunc, Roskilde, Denmark) by first adding 25 µl of culture medium, Eagle's minimum essential medium supplemented with 4% calf serum, to each well and then adding 25 µl of each test serum, the calibrated in-house standard, and positive and negative controls to the first row (1, 16). The in-house standard, calibrated against the World Health Organization standard, was titrated on each plate, and one positive and one negative control were titrated in each test. Titrations were carried out with a multichannel pipette and with 25-µl volumes. The samples were titrated in twofold dilution steps of 1:2 to 1:4,096 in 12 wells. Twenty-five microliters of the appropriate poliovirus suspension, containing a predetermined amount of virus (75 to 100 50% tissue culture infective doses), was added to each well. The poliovirus strains used were the same as those in the vaccine (see above). Binding occurred at 37°C for 3 h. The cell suspension of GMK cells (50,000 cells per ml) was added in a volume of 200 µl per well, and the plates were incubated at 37°C for 6 days. Readings were undertaken by microscopic examinations of the CPE. The lowest serum dilution completely inhibiting virus CPE was used as the endpoint, and the titers were expressed in international units per milliliter against the calibrated in-house standard.

Test sera. One hundred randomly selected, consecutive serum samples (a kind gift of Margareta Böttiger, National Bacteriological Laboratory) from a population study of poliovirus immunity in Sweden were titrated by the NT and PoBI test. In addition, 20 guinea pig serum samples from the routine vaccine control that had been tested by NT performed in fourfold dilution steps (2) were tested by the PoBI test.

Statistical analysis. Simple regression analysis was used to compute the correlation coefficient (r) between the log values of antibody concentrations ob-

tained by the NT and the PoBI assay (with software StatView). A probability (P) value of <0.05 was considered significant.

RESULTS

Establishment of the PoBI test. Optimal dilutions of the capture and indicator antibodies were established by a checkerboard titration of serial dilutions of antibodies against a fixed amount of D antigen for the three poliovirus types. The dilutions chosen were those giving the greatest optical density (OD) differences between the antigen and the buffer control. As indicator antibodies, nine different monoclonal antibodies were tested, and those giving the greatest OD differences were chosen. Incubations at 4°C were compared with those at 37°C in all steps, and 37°C was found to give steeper curves. For practical reasons, an overnight incubation period had to be used, so various incubation times for the serum virus neutralization in the first step and for the antigen binding to the capture antibody in the second step were investigated. The incubation of the serum-antigen mixture in the first step beyond 3 h did not result in a steeper curve but increased the nonspecific background. Overnight binding in the second capture step gave a steeper curve than binding for 3 h and was therefore chosen in the assay.

Sensitivity, specificity, and reproducibility of the PoBI test. Sensitivity was dependent on the antigen amount (Fig. 2). Decreasing the amount of antigen resulted in an increase of sensitivity but also in a decreasing slope of the titration curve, giving less clear-cut results. The highest sensitivity that could be achieved was 0.015 to 0.03 IU/ml for all three poliovirus types. For poliovirus types 1, 2, and 3, antigen amounts of 1.3, 0.13, and 0.53 DU/ml, respectively, generally covered the range of 0.06 to 64 IU/ml.

The specificities of the assay systems were established by titrations of monovalent antigens within the homologous and heterologous ELISA systems, with OD values of >1.0 at A_{405} for the homologous and <0.1 at A_{405} for the heterologous virus types.

A determination of the interassay variations for 10 titrations of the same sample gave a coefficient of variation of 54%, similar to the 61% for NT. In no case was the difference greater than one dilution step. A minor investigation of the intra-assay variations of five analyses of one sample on the same plate resulted in a determination of the titer at the same concentration.

Comparison of antibody determination by the NT and PoBI test. The results of titrations of 100 human serum samples against the three poliovirus types are shown in Fig. 3. Excellent correlations with correlation coefficients of $r > 0.95$ were found for all three types over the entire range of antibody concentrations. The correlation coefficients for types 1, 2, and 3 were $r = 0.974$, 0.968 , and 0.978 , respectively ($P < 0.001$ for all three comparisons).

Since many assays lack the sensitivity of the NT at low antibody concentrations, we have analyzed separately the results for the 58 determinations of antibody titers in the lowest range of 0.015 to 0.5 IU/ml (Fig. 4). Within the low titer ranges, the correlation coefficient was also found to be high: $r = 0.912$.

Varying the conditions of the PoBI test. Live, monovalent poliovirus preparations were titrated in parallel with inactivated preparations and gave the same antigen titration curves. A comparison of a limited number of guinea pig serum samples titrated for vaccine control by the routine NT in fourfold dilution steps gave a correlation coefficient of 0.885, indicating that the PoBI test can be used for antibody determinations in different species. Also, limited investigations indicated that the

FIG. 2. Effects of the viral antigen amounts on the slopes of the titration curves and the sensitivities of the assay in test samples (open circles, sample 1, 0.125 IU/ml; filled circles, sample 2, ≤ 0.06 IU/ml) in relation to a standard (filled boxes, reference serum, 1 IU/ml). Solid vertical lines, values of 1.0 IU/ml obtained from the reference serum; dotted lines, cutoffs for valid OD increases.

system could work with polyclonal, neutralizing antiserum as the indicator antibody.

DISCUSSION

The PoBI test has been shown in this study to give results that correlate excellently with antibody concentrations of all three types of poliovirus obtained by the conventional in vitro NT in tissue cultures.

The PoBI test has a number of advantages over the NT with tissue cultures. First of all, it does not require the use of live virus, which renders the test less reproducible, represents a health risk, and necessitates safe and sterile tissue culture facilities. The equipment needed for the PoBI test, a 37°C thermostat and an ELISA reader for 96-well microplates, is

FIG. 3. Comparison of results obtained by the PoBI test and conventional NT for antibodies to poliovirus types 1, 2, and 3 in 100 human serum samples. The sizes of the circles indicate the extent of overlapping values.

generally available. The assay is easy to perform and gives results within 24 h instead of 6 days. The antigen, inactivated virus, has a longer and more stable shelf-life than a virus suspension in tissue cultures. Although not investigated, it is

FIG. 4. Correlation between the NT and PoBI test for the three poliovirus types at low antibody concentrations of ≤ 0.015 to 0.5 IU/ml. The sizes of the circles indicate the extent of overlapping values.

also reasonable to assume that the PoBI test can be further simplified and shortened by using direct-conjugated indicator monoclonal antibodies.

The mechanism for the assay remains unclear. Hendriksen et al. (3) speculated that the effect would be specific for tetanus toxin, but this seems unlikely since the principle of the assay was shown to be applicable to a virus. Two mechanisms for the assay are plausible. (i) A coating of the major, neutralizing epitopes of the virus by antibodies in the first step of the assay is followed by a failure of the capture antibody to recognize or to firmly bind the virus-antibody complex, and the complex is washed away in the ensuing washing step or (ii) the virus-antibody complex is recognized and firmly bound by the capture antibody in the second step, but the neutralizing antibodies in the test sample have covered the target of the indicator antibody, and the indicator antibody is washed away in the ensuing washing step.

The PoBI test had such a high degree of correlation to the NT that one might speculate that both assays measure the same kind of antibodies. On a more theoretical basis, the most likely mechanism for the assay is the lack of firm binding of the virus-antibody complex, according to the first model above. If this is the case, then a close correlation between the PoBI test and the NT is to be expected. If, however, the principle of the test is the second model above, then it must be borne in mind that the use of only one monoclonal antibody will identify antibodies to this neutralizing epitope. Although the major neutralizing epitopes were used for monoclonal antibody tracing, other neutralization sites exist in polioviruses (6).

We chose to investigate the poliovirus system since identifying and quantifying antibodies to the three types of poliovirus in the population is an important part of evaluating the effects of immunization programs. The assay has now been applied to a bacterial toxin (3), to an enveloped virus (4), and to the unenveloped poliovirus (this study). Therefore, the principle of this assay should have a broad applicability. Since this type of assay would in many model systems represent a major improvement in safety, rapidity, and simplicity of comparison with the conventional NT, we hope that our results will encourage others to test the feasibility of using the principle of the test for other viruses.

In conclusion, an ELISA-based antibody inhibition assay was developed for identifying and quantifying antibodies to the three poliovirus types with neutralizing monoclonal antibodies as indicator antibodies, and the results of this method showed

excellent correlations to the conventional NT. The assay uses inactivated virus, is more rapid and less labor-intensive than NT, and has the potential to be generally applicable to other model systems.

REFERENCES

1. Albrecht, P., G. Van Steenis, A. L. Van Wezel, and J. Salk. 1984. Standardisation of poliovirus neutralization tests. *Rev. Infect. Dis.* 6(Suppl. 2):S540-S544.
2. Böttiger, M. 1984. Long-term immunity following vaccination with killed poliovirus vaccine in Sweden, a country with no circulating poliovirus. *Rev. Infect. Dis.* 6(Suppl. 2):S548-S551.
3. Hendriksen, C. F. M., J. W. Van der Gun, J. Nagel, and J. G. Kreeftenberg. 1988. The toxin binding inhibition test as a reliable in vitro alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *J. Biol. Stand.* 16:287-297.
4. Hong, H. A., E. J. M. Rooijakkers, N. T. Ke, J. Groen, and A. D. M. E. Osterhaus. 1994. Methods for the purification of equine rabies immunoglobulin: effects on yield and biological activity. *Biologicals* 22:1-6.
5. Linde, G. A., M. Granström, and C. Örvell. 1987. Immunoglobulin class and immunoglobulin G subclass enzyme-linked immunosorbent assays compared with microneutralization assay for serodiagnosis of mumps infection and determination of immunity. *J. Clin. Microbiol.* 25:1653-1658.
6. Minor, P. D., M. Ferguson, D. M. A. Evans, J. W. Almond, and J. P. Icenogle. 1986. Antigenic structure of polioviruses of serotypes 1, 2 and 3. *J. Gen. Virol.* 6:1283-1291.
7. Miyamura, K., E. Tajiri, A. Ito, R. Murata, and R. Kono. 1974. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. *J. Biol. Stand.* 2:189-201.
8. Miyamura, K., E. Tajiri, A. Ito, R. Murata, and R. Kono. 1974. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. II. Comparison with the rabbit skin method and practical application for sero-epidemiological studies. *J. Biol. Stand.* 2:203-209.
9. Orenstein, W. A., L. Markowitz, S. R. Preblud, A. R. Hinman, A. Tomasi, and K. J. Bart. 1986. Appropriate age for measles vaccination in the United States. *Dev. Biol. Stand.* 65:13-21.
10. Osterhaus, A. D. M. E., A. L. Van Wezel, T. G. Hazendonk, F. G. C. M. Uytde Haag, J. A. A. M. Van Asten, and B. Van Steenis. 1983. Monoclonal antibodies to polioviruses. *Intervirology* 20:129-136.
11. Osterhaus, A. D. M. E., A. L. Van Wezel, B. Van Steenis, G. A. Drost, and T. G. Hazendonk. 1981. Monoclonal antibodies to polioviruses. *Intervirology* 16:218-224.
12. Simonsen, O., M. W. Bentzon, and I. Heron. 1986. ELISA for routine determination of antitoxic immunity to tetanus. *J. Biol. Stand.* 14:231-239.
13. Van der Marel, P., A. G. Hazendonk, and A. L. Van Wezel. 1981. D-antigen determination in poliovaccine production: comparison of gel diffusion and ELISA methods. *Dev. Biol. Stand.* 47:101-108.
14. Wood, D. J., and A. B. Heath. 1992. The second International Standard for anti-poliovirus sera. Type 1, 2 and 3. *Biologicals* 20:203-211.
15. Wood, D. J., and A. B. Heath. 1992. Comparability of poliovirus neutralizing antibody tests. *Biologicals* 20:293-300.
16. World Health Organization. 1993. Standard procedure for determining immunity to poliovirus using the microneutralization test. WHO/EPI/GEN 93.9.