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Evaluation of a Poliovirus-Binding Inhibition Assay as an Alternative to the Virus Neutralization Test

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An enzyme-linked immunosorbent assay (ELISA)-based poliovirus-binding inhibition (PoBI) test to detect and quantify antibodies to polioviruses was optimized and evaluated for use in population studies as an alternative to the virus neutralization test (NT) in tissue culture. The sensitivities of the inhibition ELISA compared with the NT in an inactivated poliovirus vaccine (IPV)-vaccinated population were 98.6, 97.4, and 92.1% for serotypes 1, 2, and 3, respectively. The specificities of the PoBI test, as determined with sera from nonvaccinated persons, were also high for all three serotypes (99.0, 95.8, and 100%, respectively). Antibodies to other enteroviruses did not cross-react in the serotype 1 and 3 PoBI, and only low levels of cross-reactivity were found for serotype 2. We found high correlations between the PoBI and NT titers for serotypes 1 and 2 in IPV-vaccinated blood donors (0.97 and 0.95), in oral poliovirus vaccine (OPV)-vaccinated blood donors (0.91 and 0.95), and in naturally immune persons (0.90 and 0.87). The correlation coefficient for serotype 3, however, was significantly lower in OPV-vaccinated blood donors (0.73) and in naturally immune persons (0.76) than in IPV-vaccinated persons (0.94; $P < 0.01$). These results indicate that the antibody response to serotype 3 poliovirus in IPV recipients is different from that in OPV recipients and naturally infected persons. We conclude that the PoBI test is a suitable alternative to the NT for estimating the seroprevalence of neutralizing antibodies to poliovirus, especially in large-scale population studies.

Poliovirus neutralizing antibodies in serum are sufficient for protection against paralytic disease (2, 4, 12, 16). These neutralizing antibodies, thought to be predominantly of the immunoglobulin G (IgG) isotype, prevent poliovirus from reaching the central nervous system (5, 11). The neutralization test (NT) is used as the standard for measuring immunity to the three serotypes of poliovirus after vaccination or natural exposure. Advantages of the NT are high sensitivity and specificity and high acceptance. The NT has been chosen by the World Health Organization as the reference method for determining immunity against poliovirus (19). However, the need to use cell culture and the long duration of the test (up to 6 days) make the NT expensive and less suitable for large-scale screening of populations for protection against poliomyelitis. In addition, in view of the probable eradication of poliovirus in the near future, the use of live (wild-type) polioviruses in laboratory research and diagnostic assays (such as NT) will be discouraged or prohibited, and alternative methods for immunosurveillance of populations will be needed. Recently Edevag et al. (1) described an inhibition enzyme-linked immunosorbent assay (ELISA) for the detection of neutralizing antibodies using inactivated polioviruses as antigen. With this inhibition ELISA (poliovirus-binding inhibition test [PoBI]), a high correlation with the standard NT was found for a small set of sera in a pilot study. The specificity of the assay was not fully evaluated. Since the PoBI test is a promising alternative to the NT, we have optimized and evaluated the assay for use as a replacement of the NT in large-scale population studies. In order to do so, we tested sets of sera from (i) persons vaccinated with an inactivated poliovirus vaccine (IPV) or live attenuated oral poliovirus vaccine (OPV), (ii) persons with documented poliovirus infection, (iii) a known seronegative population, (iv) rabbits, each immunized with one of 41 different enteroviruses, and (v) a cross-sectional epidemiological survey aimed at evaluation of the national vaccination program in the Dutch population.

MATERIALS AND METHODS

PoBI. The PoBI test was performed according to the method described by Edevag et al. (1) with modifications. The PoBI test was done in two steps. For the first step (preincubation of serum and virus), microtiter cell culture plates (N655180; Greiner, Alphen aan den Rijn, the Netherlands) were blocked with 150 μl of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 37°C. Twofold dilutions of serum samples were made directly into the wells (75 μl/well, 1:2 to 1:4,096) in dilution buffer (PBS with 0.5% Tween 20, 0.5% BSA, 0.5 M NaCl). Monovalent, inactivated vaccine virus produced at the National Institute of Public Health and the Environment (RIVM) was used as antigen. The formaldehyde-inactivated poliovirus was added to each well at concentrations of 20, 4, and 16 D-antigen units/ml for serotypes 1, 2, and 3, respectively, in a volume of 75 μl per well. The concentration of D antigen was quantified with a direct ELISA (18). Poliovirus serotype 1 was Mahoney, serotype 2 was MEF, and serotype 3 was a Saukett strain. Serum-virus mixtures were incubated for 2 h at 37°C.

For the second step, Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with the IgG fraction of bovine antipoliovirus hyperimmune serum in dilutions of 1:500 for serotypes 1 and 2 and 1:250 for serotype 3 in 0.04 M carbonate-bicarbonate buffer, pH 9.6 (overnight at 4°C). Plates were blocked with 100 μl of 0.5% BSA in PBS for 1 h at 37°C. After blocking, 100 μl of the preincubated serum-virus mixture was transferred to the ELISA plate and incubated for 2 h at 37°C. For detection of bound antigen, serotype-specific monoclonal antibodies—type 1 (14D2E9, 1:3,000), type 2 (6-15C6, 1:10,000), and type 3 (2-13D9, 1:10,000)—in dilution buffer were added for 1 h at 37°C (13). The monoclonal used for serotype 1 was directed against site 2a, and the monoclonals used for serotypes 2 and 3 were directed against antigenic site 1, of the corresponding poliovirus capsid. Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, Zwijndrecht, The Netherlands) was subsequently added in a dilution of 1:500 and incubated for 1 h at 37°C. The substrate *p*-nitrophenylphosphate (Sigma) at a concentration of 1 mg/ml in 0.1 M glycine buffer (pH 10.4) was incubated at room temperature for 30 min. Plates were read at 405 nm by use of a Microwell System 510 spectrophotometer (Organon Teknica, Eindhoven, The Netherlands). For the evaluation of the PoBI test, serum samples were considered positive if a reduction in extinction of ≥50% was reached. The reciprocal of the first serum dilution that was positive in the inhibition test was taken as the

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TABLE 1. Cross-reactivity of sera from rabbits immunized with different enteroviruses in the PoBI test and the NT

Rabbit reference antiserum	NT titer of antibody to:			PoBI titer of antibody to:		
	Serotype 1	Serotype 2	Serotype 3	Serotype 1	Serotype 2	Serotype 3
Poliovirus						
Serotype 1	20,480	10	<10	4,096	16	4
Serotype 2	10	81,920	<10	64	16,384	4
Serotype 3	<10	<10	40,960	8	8	2,048
Coxsackievirus B serotypes 1–6	<2	<2	<2	<2	<2	<2
Echovirus						
Serotypes 1–6, 15–19, 24–27, 29–33	<2	<2	<2	<2	<2	<2
Serotypes 7–9, 11–14, 21–23	<2	<2	<2	<2	2	<2
Serotype 20	<2	<2	<2	<2	8	<2
Enterovirus						
Serotypes 68, 71	<2	<2	<2	<2	4	<2
Serotypes 69, 70	<2	<2	<2	<2	<2	<2

titer of the test sample. In each assay a standard in-house reference serum with known titers was included. Optimal dilutions of the coat, detector monoclonal antibodies, and conjugate were established by checkerboard titrations.

NT. Poliovirus neutralizing antibody titers of sera were determined in the standard NT as recommended by the World Health Organization (19) by using the virus strains Mahoney (serotype 1), MEF (serotype 2), and Saukett (serotype 3) as challenge viruses. In brief, serial twofold dilutions of sera to be tested and 100 50% cell culture infective doses of virus were incubated in 96-microwell plates at 37°C for 3 h. After the incubation, 1.75×10^4 HEp-2C cells were added per well. After 6 days of incubation at 37°C the plates were read. The titers are expressed as the reciprocal of the highest dilution showing complete neutralization of the cytopathic effect of 100 50% cell culture infective doses. Samples were considered positive if NT titers were ≥ 8 ($\log_2$ titer, 3).

Serum samples. For evaluation of the PoBI test, the following groups of sera were examined. (i) Sera were obtained from IPV-vaccinated blood donors in The Netherlands ($n = 26$) and from OPV-vaccinated blood donors in Belgium ($n = 42$). (ii) Sera were obtained from a group of nonvaccinated elderly persons (age, 52 to 85 years) in The Netherlands that had been given a single dose of IPV ($n = 47$). This group was presumably naturally exposed at a young age, when poliovirus was endemic in The Netherlands (8). Sera were collected at the time of IPV vaccination, and at 1 and 4 weeks thereafter. (iii) Negative-control sera were obtained from nonvaccinated children ($n = 96$) from a population which refuses vaccination for religious reasons. None of these serum samples had detectable neutralizing antibodies (titer, <2) against poliovirus. In addition, all sera were also negative for antibodies to other components of the vaccine cocktail (diphtheria and tetanus toxoid) that is used in routine immunization of children in The Netherlands. (iv) Serum samples were obtained from rabbits hyperimmunized with either poliovirus serotype 1 (Brunhilde; NT titer: type 1, 20,480; type 2, 10; type 3, <10), serotype 2 (MEF-1; NT titer: type 1, 10; type 2, 81,920; type 3, <10), or serotype 3 (Saukett; NT titer: type 1, <10, type 2, <10; type 3, 40,960) or with other enteroviruses (coxsackievirus B serotypes 1 to 6, echovirus serotypes 1 to 9, 11 to 27, and 29 to 33, and enterovirus serotypes 68 to 71). (v) A total of 785 serum samples were obtained from a cross-sectional epidemiological survey in the Utrecht Province, The Netherlands, aimed at evaluation of the national vaccination program.

Statistical methods. Regression analysis was used to determine coefficients of correlation between results obtained by the PoBI test and neutralization titers. *P* values of <0.01 were considered significant.

RESULTS

Optimization and properties of the PoBI test. To be able to reduce the duration of the PoBI test, we compared different incubation times. Preincubation of the serum-virus mixture could be reduced to 2 h at 37°C, instead of an overnight incubation at 37°C, without loss of sensitivity or changes in PoBI titers. Incubation times of the detecting monoclonal antibody and conjugate were reduced to 1 h at 37°C without influencing the outcome of the assay.

The PoBI configuration (serum-virus preincubation) was compared to direct binding of the antigen to the IgG in the ELISA plates. The PoBI titers were found to be 4- to 16-fold higher when the preincubation step was performed, and the correlation with NT was higher (results not shown).

Specificity of the PoBI test. When the previously described protocol (1) was used, 5.2, 9.4, and 6.3% of the negative-control samples tested positive in the PoBI test for serotypes 1, 2, and 3, respectively. The specificity of the assay was increased with the addition of 0.5 M NaCl in the ELISA dilution buffer to reduce aspecific binding, and this modification was used throughout the rest of the study. In this assay format, the specificity was improved to 99.0, 95.8, and 100% for serotypes 1, 2, and 3, respectively. Different blocking agents (BSA, fetal calf serum, milk and powder) had no influence on false-positive results with sera from known seronegative donors. False-positive signals were strongly reduced or disappeared after $(NH_4)_2SO_4$ precipitation of the sample, indicating that the signal was not caused by cross-reacting IgG in the serum sample (data not shown).

Sensitivity of the PoBI test. The sensitivity of the PoBI test was determined with sera from IPV- and OPV-vaccinated blood donors and from naturally immune older persons (before and after IPV vaccination). Overall, the sensitivity was 99.5, 100, and 96.2% for the serotype 1, 2, and 3 assays, respectively. The sensitivity of the PoBI test is dependent on the NT titer and was lowest for sera with low levels (titer, ≤16) of neutralizing antibodies (95, 100, and 75% for serotypes 1 to 3, respectively). The sensitivity increased to 100% at neutraliza-

TABLE 2. Correlation of the PoBI test and the NT for groups of persons with vaccine-induced or natural immunity

Group (*n*)	Correlation[a]		
	Serotype 1	Serotype 2	Serotype 3
Blood donors			
IPV vaccinated (26)	0.97	0.95	0.94
OPV vaccinated (42)	0.91	0.95	0.73*
General population (747)	0.89*	0.89	0.84
Naturally immune (47)	0.90	0.87	0.76*
1 wk[b] (47)	0.80	0.79	0.84
4 wk[b] (47)	0.79	0.76	0.84

[a] *, significantly different from the corresponding value for IPV-vaccinated blood donors ($P < 0.01$).

[b] After IPV vaccination.

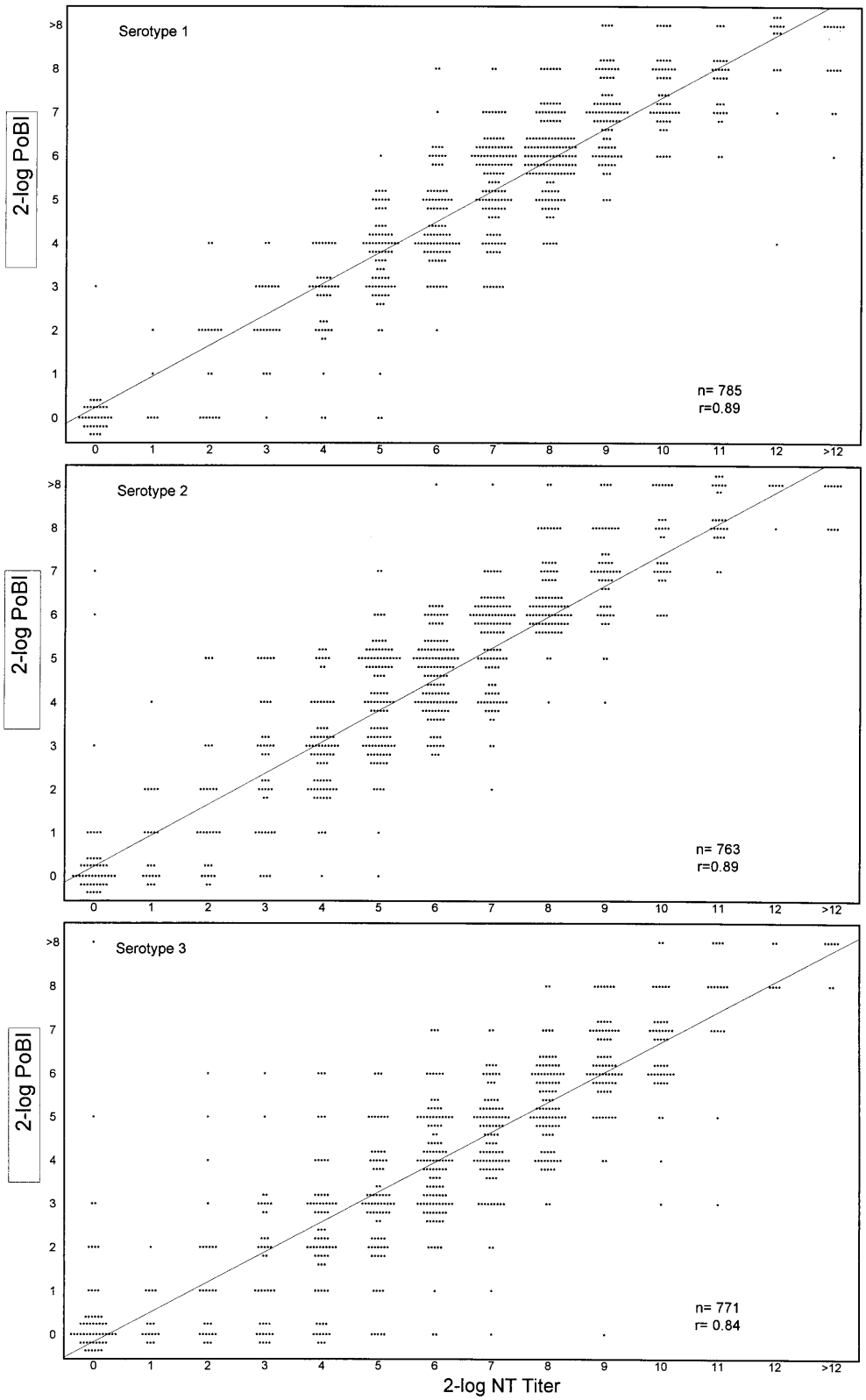

FIG. 1. Correlation between the NT and PoBI titers (expressed as $\log_2$ values) for the three poliovirus serotypes in a cross-section of different age groups in the general population.

tion titers of 16 and 64 for serotype 1 and 3, respectively (data not shown).

Cross-reactivity of the PoBI test. Hyperimmune rabbit sera were used to check for possible cross-reactivity between antibodies to poliovirus and other enteroviruses in the PoBI test (Table 1). Sera to other enteroviruses (coxsackievirus B serotype 1 to 6; echovirus serotypes 1 to 9, 11 to 27, and 29 to 33; and enterovirus serotypes 68 to 71) did not react in the PoBI test for serotypes 1 and 3. In the serotype 2 test low levels of cross-reactivity were observed with 13 of the 41 antisera that were tested. In addition, moderate levels of cross-reactivity were detected between the polioviruses in the PoBI test and the NT (Table 1).

Correlation between the PoBI titer and the NT. We found the same high correlation between the NT and the PoBI test as described by Edevag et al. (1) with serum samples from IPV-vaccinated persons (0.97, 0.95, and 0.94 for serotypes 1, 2, and 3, respectively). In OPV-vaccinated blood donors and naturally immune persons the coefficient of correlation between the NT and the PoBI test for serotypes 1 and 2 was high (Table 2). However, for serotype 3 the correlation coefficient was significantly lower in OPV-vaccinated blood donors (0.73) and naturally immune persons (0.76) than in the IPV-vaccinated blood donors ($P < 0.01$) (Table 2). The correlation between PoBI and NT titers was similar for sera collected at different time points after booster vaccination of naturally immune persons vaccinated with IPV (Table 2).

Evaluation of immunity in the general (IPV-vaccinated) population. A total of 785, 763, and 771 sera were examined by the PoBI test and the NT for serotypes 1, 2, and 3, respectively. In the general population the coefficients of correlation between the NT and the PoBI test were 0.89, 0.89, and 0.84 for serotypes 1, 2, and 3, respectively, significantly lower than the correlation between the NT and the PoBI test using sera from IPV vaccinees for serotype 1 (Table 2). No differences in the correlation between the PoBI test and the NT were found for the different age groups (data not shown). From the regression line, the PoBI titer that corresponds to a titer of 8 in the NT was calculated to be 4 (Fig. 1). Therefore, sera with PoBI titers of <4 were considered negative. The sensitivity of the PoBI test was high for all three serotypes: 98.6, 97.4, and 92.1% for serotypes 1, 2, and 3, respectively. The positive predictive values of the PoBI test in the general population were 0.98, 0.97, and 0.97 for serotypes 1, 2, and 3, respectively. Specificity in this group was 80.3% for serotype 1, 82.0% for serotype 2, and 79.8% for serotype 3. The negative predictive values were 0.83 and 0.82 for types 1 and 2, respectively, and only 0.61 for type 3. With both assays, a normal distribution of titers was seen in addition to a group of seronegative results (PoBI and NT titers of <4 and <8, respectively) for all three serotypes (Fig. 2). PoBI titers were generally twofold lower than the standard NT titers. The total numbers of seronegative individuals in the general population were estimated to be 7.5, 12.4, and 17.4% with the PoBI test and 7.8, 12.5, and 13.3% with the NT test for the three serotypes, respectively. These percentages were not significantly different (at a P level of <0.01). The PoBI test proved to give a good estimate of the total number of seronegative subjects within the different age groups in the general population for all three serotypes (Fig. 3). The PoBI test showed patterns of seroprevalence in the different age groups similar to those in the NT for all three serotypes.

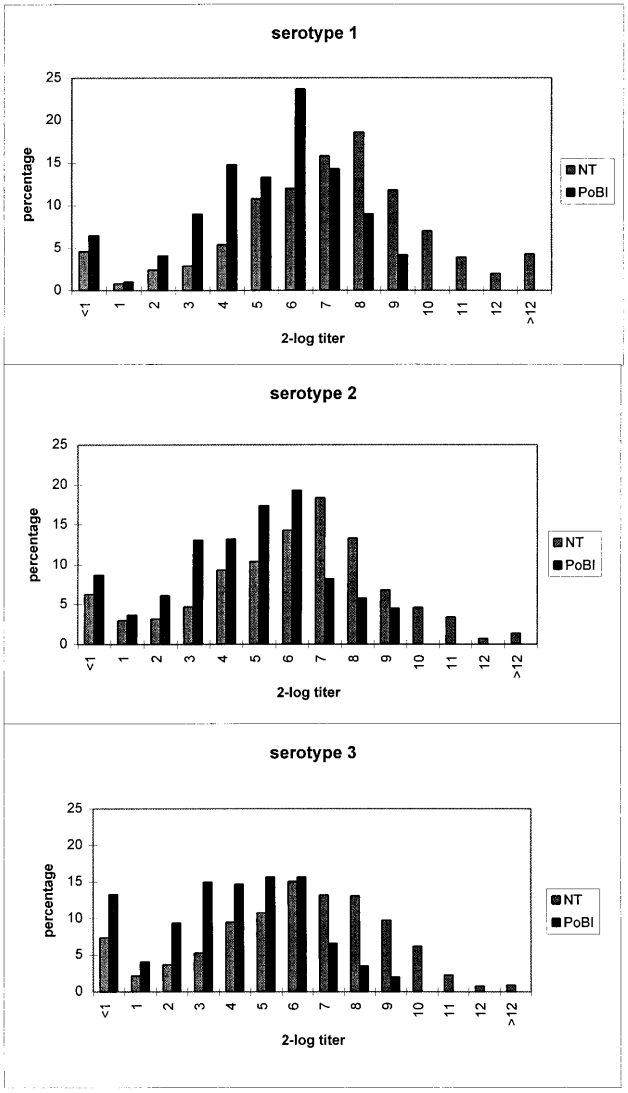

FIG. 2. Frequency distribution of NT and PoBI titers (expressed as $\log_2$ values) in a cross-section of different age groups in the general population. Results are presented as percentages of persons positive per titer. The highest serum dilution tested was 1:256 ($\log_2$ 8) in the test and 1:4,096 ($\log_2$ 12) in the NT.

DISCUSSION

Previously described ELISAs for measuring protective antibodies to poliovirus could not compete with the very sensitive NT (6, 7, 10, 17). With a direct ELISA format for serotype 1, Simhon et al. (17) obtained positive predictive values between 0.82 and 0.91 but found high numbers of false-negative results, and negative predictive values were low (between 0.29 and 0.55). Hagenaars et al. (7) described an inhibition ELISA in which serum antibodies and labelled bovine anti-poliovirus serotype 1 competed for binding places on the bound antigen. The assay gave good correlation with the NT, but the standard NT assay was more sensitive than the inhibition ELISA.

The PoBI test, in which inhibition of the signal depends on both reduction of virus-antigen binding to the capture antibody and reduction of binding of the indicator monoclonal antibody, was found to be a suitable replacement for the NT in large-scale population studies. Although PoBI titers were generally (twofold) lower than NT titers, neutralization-positive samples can easily be identified with the PoBI test. Sensitivity and positive predictive values were high in both IPV- and OPV-vaccinated persons and in naturally exposed people. The spec-

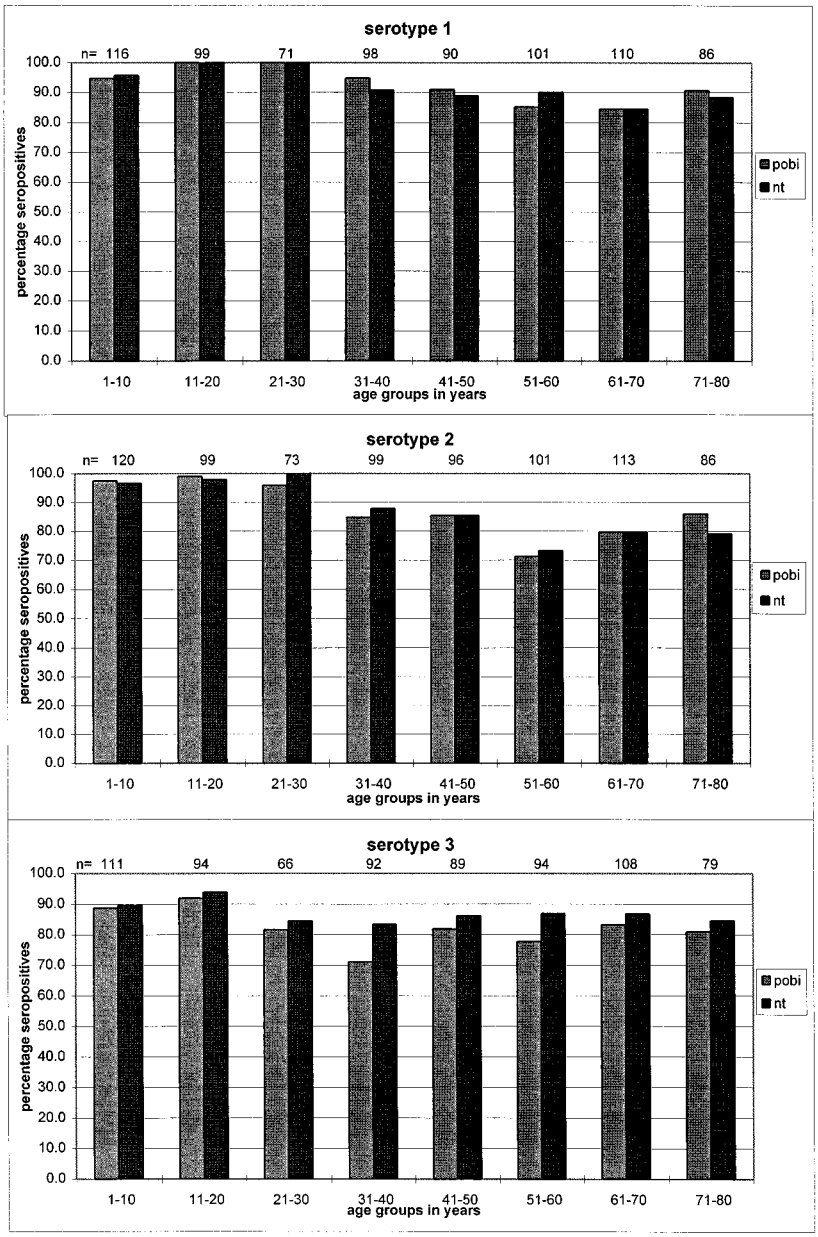

FIG. 3. Seroprevalence of poliovirus antibodies in a cross-section of different age groups in the general population as determined by PoBI and NT assays. NT titers of ≥8 and PoBI titers of ≥4 were considered positive.

ificity of the PoBI test was determined with selected sera from nonvaccinated subjects and was high (95 to 100%). In the general population, persons with low NT titers (titers of 2 and 4) are considered to be at risk of poliovirus-induced disease. Therefore, for the purpose of this evaluation, sera with NT titers of <8 were considered negative, as were sera with a corresponding cutoff titer of <4 in the PoBI test. The detection of these low NT titers in the PoBI test is responsible for the lower specificity of the PoBI test calculated from comparative serology in the population (79.8 to 82.0%) and, similarly, for the low negative predictive value for the serotype 3 assay. The question is whether NT titers of 2 and 4 are truly negative or should be considered low but specific antibody levels. Most likely, this group consists of both true negatives and low positive samples. Therefore, PoBI-negative sera should be retested by NT. The PoBI results with rabbit hyperimmune sera showed that cross-reactivity with other enteroviruses did not occur for serotypes 1 and 3 and occurred only at low levels for type 2. Since titers to enterovirus antigens as high as the levels in the hyperimmune-rabbit serum samples are not likely to be detected in the general population or in patients, this low-level cross-reactivity is not a problem for serosurveys in which the PoBI test is used.

The purpose of this seroprevalence study was to estimate the total number of seronegative individuals (as determined by the NT and by the PoBI test) within the Dutch population. A high percentage of seronegative individuals in the population indicates a potential risk for poliomyelitis outbreaks and requires active revaccination of the (age) groups at risk. The PoBI test proved to be an excellent indicator of seroprevalence in an

IPV-vaccinated population for all age groups. The two assays yielded similar estimates of the total number of seronegative individuals within the general population for all three serotypes. We conclude that the PoBI is a suitable test for seroprevalence studies of poliovirus; it is a less labor-intensive assay that is easier to perform than the NT, can be further automated, and is not dependent on visual screening of the cytopathic effect in contrast to the standard NT. This assay could be used for population screening, in combination with confirmatory testing of PoBI-negative sera by NT. By this approach, at the current prevalence of poliovirus antibodies the total number of serum samples to be examined by the NT can be reduced by 87.5%.

We obtained the same high correlation between the PoBI test and the NT as that described by Edevag et al. (1) for IPV-vaccinated subjects. However, in OPV recipients and naturally immune persons the correlation between PoBI and NT titers of antibody against serotype 3 was significantly lower and tended to be lower for the other serotypes as well. In part, this may be explained by the use of IPV (and not OPV) as antigen in the PoBI test. The greater difference observed in the serotype 3 PoBI test may be explained by a more narrow immune response against serotype 3 poliovirus compared with the other serotypes. In animals, site 1 of serotype 3 is extremely immunodominant (3, 9). Therefore, the polyclonal coat for the serotype 3 PoBI test may consist mainly of (neutralizing) antibodies to site 1 and, as a result, may be very sensitive to changes in immune response directed to site 1. In this context, it is intriguing that differences in immune responses have been observed between infection by wild-type serotype 3 poliovirus or OPV vaccination on the one hand and IPV-induced immunity. During infection with live viruses (wild-type or OPV), site 1 of serotype 3 is cleaved by trypsin during passage through the gut lumen, thereby exposing other immunogenic sites on the viral capsid (14, 15). This trypsin effect will not occur in IPV recipients, since the vaccine is given by intramuscular injection and therefore the trypsin-dependent immunogenic sites will be less well exposed to the immune system. In contrast to serotype 3, the PoBI assays for serotypes 1 and 2 are probably reactive with antibodies to more than one antigenic site, so that differences between OPV and IPV vaccinees are not detected. Future work will focus on the site specificity of the human antibody response to poliovirus. In conclusion, the newly developed PoBI test can replace the NT in large-scale population studies for determining protective levels of antibodies to polioviruses. PoBI-negative sera should be retested by the NT for confirmation of seronegativity. One of the major advantages of the PoBI test over the NT is that inactivated virus is used. In view of the ongoing eradication of poliovirus, the use of live poliovirus in diagnostic assays should be discouraged and disappear in the near future.

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