Intestinal Immunity Is a Determinant of Clearance of Poliovirus After Oral Vaccination

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Background. Response to challenge with live, attenuated, oral polio vaccine (OPV) is a measure of immunity induced by prior immunization.

Methods. Using stool samples from a study from Oman in which an initial schedule of inactivated polio vaccine (IPV) was followed by an OPV type 1 challenge, we quantitated virus shed, sequenced capsid proteins of recovered virus, and developed assays for neutralization of poliovirus and mucosal immunoglobulin A (IgA) detection.

Results. Neutralizing activity correlated with detection of polio-specific IgA in stool suspensions collected 7 days after OPV type 1 challenge. Both neutralization and IgA in stool were associated with cessation of virus shedding by day 7. Rapid development of an IgA response with cessation of shedding suggests that IPV primed for the early response to challenge. Correlation of neutralization activity and IgA detection provides evidence that polio-specific IgA intestinal antibody is a determinant of mucosal shedding/transmission and that IgA functions through neutralization of virus. In contrast, neither presence nor quantity of serum or intestinal antibody induced by IPV prior to challenge correlated with cessation of shedding.

Conclusions. These assays provide an opportunity to study other immunization schedules to gain a broader understanding of the appearance and duration of a protective mucosal response to polio vaccination.

Keywords. polio; vaccine; IgA; mucosal immunity.

Immunity to polio has 2 functions. The first is prevention of clinical poliomyelitis. Serum antibody is the protective modality that works through neutralization of virus during viremia—an essential step in establishing a central nervous system infection [1]. The second is limiting the replication of polio virus in the enteric and respiratory tracts and hence decreasing person-to-person spread of virus.

It has long been postulated that the 2 available polio vaccines—live, attenuated orally administered (OPV) and inactivated, systemically administered (IPV)—stimulate different immune responses. Both vaccines effectively induce serum antibody and are highly effective in prevention of poliomyelitis in the individual vaccine recipient. However, OPV is less immunogenic in certain tropical settings [2], and multiple doses are necessary to approach full protection [3]. Early studies by Ogra et al showed that the 2 vaccines differed in the induction of immunoglobulin A (IgA) antibody at enteric sites, with intestinal IgA found after OPV but not IPV [4]. Additional studies in which a challenge of OPV was given after an OPV or IPV series suggest that OPV induces greater intestinal immunity than IPV [5]. Most of the success of the Global Polio Eradication Initiative (GPEI) has been the result of the use of OPV; nevertheless, there are clear examples in which IPV alone has effectively eliminated wild-type virus [6].
The efforts to successfully complete the eradication of polio will depend on the use of IPV. Reasons for this are the continued spread of vaccine-derived polio strains and the long-recognized though rare occurrence of vaccine-associated paralysis from the use of OPV. The success of this change in the direction of the eradication program will depend on a clear understanding of individual and community resistance to acquisition of polio immunity afforded by each vaccine.

The rationale for thinking that either vaccine will be effective in the final stages of polio eradication has traditionally rested on examples of countries that have successfully eliminated polio through the exclusive use of either IPV (Scandinavian countries) or OPV (India being the most recent example). At a more granular level, studies of the effect of IPV and OPV on the shedding of either monovalent or trivalent OPV have suggested that although both OPV and IPV are highly immunogenic in terms of serum antibody responses, there is a greater protection afforded by OPV against viral shedding. Perhaps the most definitive information has come from a series of studies in Cuba. In that country, the unique schedule for administration of OPV in twice-yearly campaigns has allowed the clear differential effects of each vaccine to be measured [7, 8]. The data with 3 doses of IPV and a subsequent trivalent OPV challenge suggest that there is little effect of prior IPV on OPV recovery with a total OPV shed on day 7 of 3.46 log per gram of stool (95% confidence interval [CI], 3.17–3.75) with prior IPV and 3.89 log (95% CI, 3.64–4.14) in polio-naive children. The dominant shedding with a first dose of trivalent OPV is expected to be type 2, which was recovered in 87% of IPV recipients and 89% of polio-naive subjects. As the vaccine given was trivalent, the shedding of type 1 with the first dose was lower—only 17% and 19%, respectively.

The extent and duration of intestinal immunity after OPV and IPV has recently been addressed with a “Delphic” analysis and with a critical literature review. The former analysis demonstrated widely divergent impressions among the experts as to the amount and duration of intestinal immunity induced by IPV [9]. The systematic review came to the conclusion that no intestinal immunity or effect on virus shedding was seen after IPV [10].

The ability to protect against transmission is central to the decisions that lie ahead for polio eradication. Measurements of intestinal antibody responses are inherently difficult. Mucosal samples not only have the complexity of a nonhomogeneous substance but also may cause proteolytic breakdown of immunoglobulin, exhibit toxicity to and/or cause bacterial contamination of any cell-based assay system, display mixed antibody class responses, and suffer from dilutional effects of the collection methodology. Most measurements of mucosal immune responses have relied on binding measurement of immunoglobulin class responses by enzyme-linked immunosorbent assay or similar techniques. This paper reports both the development of an assay that detects an early poliovirus type 1 (PV1)–specific neutralizing response and a sensitive polio type–specific IgA immunoassay, both of which correlated with cessation of virus shedding in the stools of children in Oman who had received a type 1 (OPV1) challenge at 7 months of age after IPV at 2, 4, and 6 months of age.

**METHODS**

**Study Design and Participants**

Stool specimens for this study were obtained from a poliovirus vaccine trial in Oman [11]. Infants received IPV vaccine at 2, 4, and 6 months. A monovalent OPV1 was given at 7 months (referred to as day 0) and stool samples were collected 7 days after the challenge dose. Samples were stored at −40°C in the laboratories of the National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands. The isolates studied were those from the arm of the study in which children received the full dose of IPV; another arm of the study used a lower dose [11]. Permission was obtained from the Omani investigators to use the samples.

Serum samples were collected on days 0 and 28, and results of neutralization and serum IgA assays performed at the Centers for Disease Control and Prevention (CDC) were kindly made available for analysis in this study.

**Procedures**

**Viral Reisolation and Titration**

Virus-containing stool suspensions were diluted in Eagle’s minimum essential medium (EMEM) with 10% fetal bovine serum (FBS), for a total of six 10-fold dilutions. Dilutions were tested in quadruplicate in microtiter dishes. Twenty-five microliters of EMEM + 10% FBS was added to each test well and then 25 µL of virus dilution was added to the appropriate wells. The plates were kept at 37°C and 5% CO₂ for 3 hours. After 3 hours, 25 µL of a cell suspension of L20B cells at 3×10⁵ cells/mL was added to the test wells. Plates were incubated for 5 days at 37°C and 5% CO₂. Plates were read for cytopathic effect after 5 days. Plates were then frozen at −80°C for virus blind passage. Confluent L20B cells in microtiter plates were switched to maintenance media by discarding the growth media and adding 25 µL of EMEM + 2% FBS. Twenty-five microliters of thawed virus/medium from the original passage was added to the new plates in the same plate format. Plates were incubated for 5 days at 37°C and 5% CO₂. Plates were read for cytopathic effect after 5 days, and the virus titer was determined using the Kärber formula [12].

**Serum Antibody**

Serum neutralizing antibodies had been determined in the CDC’s entero-virus laboratory using Sabin virus strains and were shared by the GPEI to determine any relationship between the presence and quantity of serum antibody at the time of challenge with subsequent virus shedding. Serum IgA titers to PV1 were
Poliovirus OPV OPV1 was recovered by RIVM from 96 of 157 of the day 7 stools after OPV challenge [11]. Virus was reisolated from 93 of the stools initially positive at RIVM at Dartmouth. On day 7, the titer of type 1 viruses isolated was up to $10^8$ tissue culture infectious dose (TCID$_{50}$/mL) of 10% stool suspension with a geometric mean titer of 2.95 log in those shedding virus (Figure 1).

Prior studies in Oman have demonstrated the community spread of OPV [17]. Indeed, limited numbers of OPV viruses were found on day 0 (1 OPV1, 2 poliovirus type 2 [OPV2], and 1 poliovirus type 3 [OPV3]) or day 7 (1 additional OPV2), indicating some circulation of OPV prior to challenge. These samples were not excluded from the analysis.

### Statistical Analysis

Because they were not normally distributed according to the Shapiro-Wilk test of normality, associations between antibody responses and OPV recovery were evaluated using the nonparametric Spearman rank correlation test. Associations between neutralizing antibodies at day 0 and OPV recovery were also evaluated with Fisher exact test applied to binary indicators of their presence/absence. The relationship between the presence of substitutions in the VP1 antigenic epitope 1 and viral shedding titers on day 7 was examined using analysis of variance.

### RESULTS

**Virus Isolation**

Poliovirus OPV OPV1 was recovered by RIVM from 96 of 157 of the day 7 stools after OPV challenge [11]. Virus was reisolated from 93 of the stools initially positive at RIVM at Dartmouth. On day 7, the titer of type 1 viruses isolated was up to $10^8$ tissue culture infectious dose (TCID$_{50}$/mL) of 10% stool suspension with a geometric mean titer of 2.95 log in those shedding virus (Figure 1).

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**Prechallenge Serum Antibody Correlates of Virus Recovery on Day 7**

Type 1 serum neutralizing titer at the time of virus challenge (day 0) showed no correlation with recovery of OPV 1 on day 7 (Spearman correlation coefficient $-0.018$, $P = .826$; Figure 2A). Serum IgA antibody to OPV 1 was measured on 37 subjects as a marker for prior exposure to concurrently circulating OPV at the CDC. Twenty (54%) of the 7-month serum samples had low levels of IgA detected. There was no correlation between detected levels of prechallenge serum IgA and recovery of virus (Spearman correlation coefficient $-0.129$, $P = .446$).

**Prechallenge Stool Antibody Correlates of Virus Recovery on Day 7**

The day zero 10% stool suspension neutralization titers ranged from undetectable to 60% neutralization at 1:32 dilution. The detection of prechallenge stool neutralizing activity was not significantly correlated with the presence of virus in the stool on day 7, or with viral titer (Spearman correlation coefficient $-0.016$, $P = .846$; Figure 2B). The level of OPV1–specific IgA in the prechallenge stool also did not correlate with recovery or amount of virus on day 7 (Spearman correlation coefficient $-0.143$, $P = .085$; Figure 2C).

**Day 7 Stool Antibody Correlates of Virus Recovery on Day 7**

By day 7, type 1–specific stool neutralization had a distinctly different profile, with 22 stools showing antibody titers that were $>1:32$, and many $>1:128$. Thirty pairs of stools showed a $>4$-fold rise between days 0 and 7. The stools in which there was detection of neutralization at a titer of $>1:32$ on day 7 were significantly less likely to have virus recovered than stools with lower titers that overlapped with prechallenge values (odds ratio, 0.15 [95% CI, .05–.46]; Table 1). When the quantitation of virus in stool was examined, there was a significant inverse correlation between day 7 neutralization activity and...
recovered virus titers (Spearman correlation coefficient $-0.170$, $P = .039$; Figure 2D). The inhibition was specific for PV1 with virtually no responses seen to either PV2 or PV3 pseudovirus (Table 2).

The day 7 OPV1-specific IgA titers in the stool were significantly higher than prechallenge (data not shown). IgA levels were significantly inversely correlated with the amount of virus present on day 7 (Spearman correlation coefficient $-0.165$, $P = .046$; Figure 2E), and the IgA levels and neutralization activity were strongly correlated in the day 7 specimens (Spearman correlation coefficient 0.324, $P < .0001$; Figure 2F).

**Molecular Sequences of Shed OPV**

Sequencing of VP1, VP2, and VP3 demonstrated that the majority of changes were in VP1 with a single “hot spot” at amino acid position 90 within antigenic epitope 1 (Figure 3). This single site had a high substitution frequency (>25%). Because the change was both at an antigenic epitope and in a
position that might influence receptor affinity, we could not differentiate between antibody modification and host adaptation. However, the nonrandom distribution of substitutions certainly indicates that they were not due to simple infidelity of genome replication. There was a significant association between the development of this substitution and the amount of virus shed on day 7 (F(1, 145) = 11.886; P < .001), suggesting that higher day 7 virus recovery titers may be a consequence of the emergence of neutralization escape or altered viral fitness (Figure 4). As sequencing depends on recovery of virus and sampling was done on a single day, we cannot discern the relationship between duration of shedding and reversion at this site. Also noted is that the OPV type 1 pseudovirus has the capsid proteins of PV1 (Mahoney) and not that of the Sabin strain. The antigenicity of PV1 (Mahoney) and OPV type 1 (Sabin) differ [14].

DISCUSSION

An assay that enables detection of inhibition of virus shedding without the nonspecific interference of toxicity/bacterial contamination that is often seen in a classical neutralization test performed on mucosal samples would be a major advance. The assay described here using a poliovirus pseudovirus appears to offer that possibility (see further details of assay development and validation in the Supplementary Data) [18]. Its potential physiologic importance is reinforced by the inverse correlation of antibody rise with virus shedding. The demonstration that inhibition was OPV1 specific and did not inhibit OPV2 or OPV3 strains suggests that we are detecting an adaptive immune response rather than nonspecific antiviral activity of other factors present in stool. The microsphere assay data showing a correlation of inhibition with OPV1 IgA antibody implies that this inhibition may be mediated by IgA in the mucosal environment. Noted are demonstrations that nonspecific/innate responses can occur as, for example, short-term protection by type 8 rhinovirus infection against type 23 challenge [19].

A remarkable aspect was detection of inhibition as early as day 7 after challenge, raising a question of whether the development of inhibition might be enhanced by prior exposure to IPV. There is evidence that prior rotavirus exposure led to a significantly elevated IgA response within 4 days of when a candidate live rotavirus vaccine was administered [20]. The concept that IPV can prime for an early serum antibody response was reinforced by a recent study from Cuba in which a subpotent dose of IPV primed for a booster response to IPV that was seen on day 7 [7]. The correlation between neutralization and cessation of virus shedding, while statistically significant, was not complete. A potential explanation of this may be provided by a study with respiratory syncyial virus suggesting that there is a transition period of increasing mucosal antibody with still detectable virus [21]. A limitation of this study is that samples were available from only a single day after challenge.

Reanalysis of a study done in Nashville [22] provides some data on the expected duration of shedding of virus with a first dose of OPV. Type 1 virus shedding was examined on day 30 or day 60 after the first dose of OPV in those children who had

Table 1. Inverse Correlation of Virus Recovery With Level of 60% Neutralization in Day 7 Stool Suspension

<table>
<thead>
<tr>
<th>Virus Shedding</th>
<th>Neutralization ≤1:32a</th>
<th>Neutralization &gt;1:32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td>No</td>
<td>37</td>
<td>16</td>
</tr>
</tbody>
</table>

*a Titer chosen because that was the upper limit of inhibition in prechallenge stool. Odds of shedding if neutralization >1:32 = 6/16 = 0.38. Odds of shedding if neutralization ≤1:32 = 90/37 = 2.43. Odds ratio = 6/16/90/37 = 0.15. Fisher exact test (2-sided). Alternative hypothesis: true odds ratio is not equal to 1. Ninety-five percent confidence interval, .05–.46, P < .001. χ² = 15.55, df = 1, P ≤ .001.

Table 2. Increases in Polio Neutralization in Stools on Day 7 Are Largely Serotype 1 Specific

<table>
<thead>
<tr>
<th>Stool Antibody Titer</th>
<th>Day 0</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 1</td>
<td>Type 2</td>
</tr>
<tr>
<td>Undiluted</td>
<td>108</td>
<td>104</td>
</tr>
<tr>
<td>1:2</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>1:4</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>1:8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>1:16</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1:32</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1:64</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>≥1:128</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Boldface is to highlight that rises in titer only occurred to OPV1.

Figure 3. Sequence changes detected in poliovirus capsid proteins (VP1, VP2, VP3) in virus recovered on day 7.
a type 1 antibody response, a strong correlate of infection in the Nashville study (Figure 5). Although not significantly different, a lower percentage of children who were shedding OPV1 on day 7 from Oman than day 30 in Nashville, suggesting that virus shedding at the time of the first infection may be of longer duration in naive children than in IPV-primed individuals.

Differential shedding of OPV1 in children depending on vaccine received (IPV vs OPV) was shown in studies in Baltimore. On day 7, 82% of subjects shed virus after receipt of IPV and 31% after prior OPV. The time course of virus shedding suggested a sharp decrease in virus recovery by days 7 (approximately 30%) and 14 (approximately 20%) in IPV recipients [23]. Unfortunately, no stool samples remain from either the Nashville or the Baltimore studies to more definitively define differences in immunity induced by IPV and OPV with the assays described in this paper.

It is postulated that intercurrent OPV exposure in Oman was not a major confounding factor in interpreting the results, as the low levels of serum IgA detected did not correlate with recovery of virus, and IgA to OPV1 was detected only at low levels in the day 0 stool. IPV can induce some serum IgA [4]. However, it is noted, in a limited number of stool samples from young US children, that no stool neutralizing antibody was seen (Supplementary Data).

The study also reinforces the genetic plasticity of OPV when administered to humans. It has been suggested that prior IPV increases the mutational rate of subsequent doses of OPV [24, 25]. This study demonstrates that genetic adaptation was less frequent with OPV after IPV than was seen in Egypt with a 1-month challenge of a birth dose of OPV, although prolonged excretion of the birth dose cannot be excluded [26]. The importance of viral genetic changes in either adapting to the host environment and/or evading the early host immune response are suggested by the significantly higher amount of virus shed among strains that had undergone changes.

A broader application of these assays to other existing repositories or in prospective trials which contain samples that have been properly treated and stored is indicated. Further study is merited, including validation of the assays with different panels of stools, analysis of the induction of such responses by OPV, more precision in understanding the timing of this response and its correlation with cessation of virus shedding, and determination of the duration of such a response after vaccine exposure. Most critical may be whether this is a memory response triggered by IPV, which would predict that shedding of live poliovirus after exposure is of shorter duration as a result of prior IPV immunization, even if diminution in titer shortly after live poliovirus exposure is minimal. This would lend credence to the use of IPV in the final stages of polio eradication and to the hope that it would diminish the risk of circulating vaccine-derived polio or reemergence of wild-type virus from unrecognized reservoirs.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.