Poliovirus-Specific Intestinal Antibody Responses Coincide with Decline of Poliovirus Excretion

Sanna Valtanen, Merja Roivainen, Liisa Piirainen, Mirja Stenvik, and Tapani Hovi

Antibody responses to poliovirus type 3 were studied in fecal samples of 66 children immunized with 3 doses of enhanced-potency inactivated poliovirus vaccine (E-IPV), followed by 1 dose of monovalent oral poliovirus vaccine (OPV, type 3 Sabin). One fecal sample taken before OPV vaccination and 9 collected thereafter were tested for neutralizing antibodies by a microneutralization assay and for class-specific responses by heavy chain–capture radioimmunoassays. Both neutralizing antibody and IgA responses usually occurred during the second week and coincided with ceasing of virus excretion or a decrease in the excreted virus titer. Half of the vaccinees had received a trypsin-modified E-IPV, but their responses did not differ from those of children immunized with the regular E-IPV. These results are in agreement with the view that an intestinal antibody response, mainly consisting of IgA, may be involved in the ceasing of a primary poliovirus excretion.

The enterovirus genus comprises polioviruses, coxsackieviruses, echoviruses, and enterovirus serotypes defined by numbers 68-91, all of which inhabit the human alimentary tract and are usually transferred through the fecal-oral route. The natural course of infection is best known for polioviruses, the causative agents of paralytic poliomyelitis [1]. Although a neutralizing antibody response can be demonstrated in the circulation by 10–14 days of infection, replication of the virus continues in the gut-associated lymphoid tissue for several weeks, and excretion of the virus into feces may persist for months. Host defense factors that eventually eliminate the virus have remained obscure.

Administration of the live attenuated oral poliovirus vaccine (OPV) is known to induce a good serum antibody response and a proper intestinal immunity [2–4]. A disadvantage of OPV is vaccine-associated paralytic poliomyelitis, which is sometimes, although very infrequently, caused in vaccine recipients or their contacts [5]. The parenterally administered inactivated poliovirus vaccine (IPV) is safe and induces good serum immunity [3], but the resistance to infection in the mucosal surfaces is poor compared to that following OPV or natural infection [3, 6], even with the modern enhanced-potency preparations (E-IPV) [7–11].

The antigenic site distribution of antibody specificities induced by IPV is different from those seen after OPV or natural immunizations, especially as regards poliovirus type 3 (PV3) [12–14]. The relative overrepresentation of PV3-induced antibodies targeted to the loop between β-strands B and C (BC-loop) of the VP1 capsid protein can be eliminated in mice by treating the immunogen with trypsin that selectively cleaves the BC-loop [15, 16]. This led us to test the idea that IPV-induced intestinal immune response in humans could be improved by treating the PV3 component in the IPV-vaccine with trypsin in advance [17, 18]. A clinical trial was carried out with groups of infants immunized with either E-IPV or trypsin-modified E-IPV (TryIPV) and challenged with a dose of orally administered PV3/Sabin. The vaccine groups showed, however, no significant difference in the intestinal immunity as judged by the length and amount of excreted challenge virus [18]. In the present study, we have compared the groups as regards kinetics of development of fecal antibodies against PV3 between 2 and 42 days after the OPV challenge, by measuring both neutralizing antibodies and class-specific IgA, IgG, and IgM responses. We also compared these antibody responses child-by-child with the excretion of PV3 to determine whether the antibody response in the intestines would be temporally associated with ceasing of virus excretion.

Subjects, Materials, and Methods

Immunizations and sample collection. The clinical trial was carried out at 2 municipalities near Helsinki. Children enrolled in the study (n = 100) were randomized in 2 groups to receive, at 4, 6, and 12 months, either a dose of the regular E-IPV (40, 8, and 32 D-antigen units [DU] of inactivated PV1, PV2, and PV3, respectively) or a modified version of it, TryIPV, with the type 3 com-
Table 1. Fecal neutralizing antibody responses in immunized children challenged with a dose of oral monovalent poliovirus vaccine type 3.

<table>
<thead>
<tr>
<th>Vaccine group (n), antigen</th>
<th>No. (%) of responders</th>
<th>Peak titer (percentile)</th>
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<tbody>
<tr>
<td></td>
<td>Median</td>
<td>75th</td>
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<tr>
<td>E-IPV (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact PV3</td>
<td>23 (64)</td>
<td>64</td>
</tr>
<tr>
<td>Trypsin-treated PV3</td>
<td>22 (61)</td>
<td>128</td>
</tr>
<tr>
<td>TrypIV PV3 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>17 (57)</td>
<td>32</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>17 (57)</td>
<td>32</td>
</tr>
</tbody>
</table>

NOTE. Antigen, type of virus preparation used in assay; E-IPV, enhanced-potency inactivated poliovirus vaccine; n, number of children in group; PV3, poliovirus type 3; TrypIV, trypsin-modified E-IPV; responder, vaccinee with >1 postchallenge fecal specimen positive in corresponding RIA. There was no significant difference in pairwise comparisons between vaccine groups or between antigen groups (χ², P > .18). Peak titer, highest titer recorded in any examined fecal specimen of a given vaccinee; median and upper 75th and 90th percentiles, respectively, are given for each group. There was no significant difference in pairwise comparisons between vaccine groups or between antigen groups (Mann-Whitney, P > .083).

Vaccines were prepared by the National Institute for Public Health and Environment, Bilthoven, The Netherlands. At 18 months, the children received a dose of monovalent OPV (5 × 10⁸ TCID₅₀ of type PV3/Sabin, a gift from SmithKline Beecham Vaccines, Rixensart, Belgium). Fecal samples for analyzing virus excretion were collected at day 0 and at 2, 5, 8, 12, 16, 21, 28, 35, and 42 days after the challenge.

The specimens were processed the same day they arrived at the laboratory. A fecal suspension at 1:6 (i.e., ~1 cm³ of fecal sample mixed with 5 mL of Eagle MEM, supplemented with 2% fetal calf serum [FCS]) was prepared by shaking with glass beads in a Vortex-mixer and clarified by centrifugation at 2000 rpm for 10 min. The supernatant was extracted with chloroform (4 + 1 v/v). After centrifugation for 15 min at ~1200 g, the supernatant was used for culture [18], and the remaining part was frozen to −20°C.

Virus quantitation and identification. Concentration of infectious virus in the fecal suspension supernatants was determined by the endpoint dilution method in L20B [19] and GMK cells on microwell plates as described in detail by Piirainen et al. [18]. In the following, only data obtained in the L20B cells will be presented. Positive cultures were harvested and stored at −20°C until further testing. Isolates were typed by neutralization with a serotype-specific rabbit antiserum. All specimens primarily showing no cytopathic effect (CPE) were examined in a blind passage. The quantity of virus in the specimens was calculated from the highest dilution showing 50% CPE and is expressed as 50% cell culture-infecting dose (CCID₅₀)/cm³ of fecal specimen.

Neutralizing antibodies. For determination of neutralizing (NT) antibodies in the fecal suspensions, the suspensions were heated for 30 min at 56°C to inactivate viruses. NT antibodies were measured by a modification of the standard microneutralization assay [20]. In brief, serial 2-fold dilutions (1:2–1:512) of fecal suspension (1:12–1:3072 dilution of the original feces) were prepared in MEM, supplemented with antibiotics and 2% FCS; 100 CCID₅₀ of PV3 strain Sabin, either intact or trypsin-treated [12], was added, and the mixtures were incubated at 37°C for 2 h and subsequently overnight at room temperature. The next day, the mixtures were inoculated onto ready-made monolayers of Vero cells in microwell plates and incubated at 36°C for 1 h, followed by removal of the remaining inoculum and addition of 200 μL of maintenance medium (MEM, supplemented with antibiotics and 2% FCS). Cultures were incubated for 5–7 days at 36°C in 5% CO₂. Dilutions showing complete inhibition of virus-induced CPE were scored positive, and the corresponding reciprocals were taken as NT titers.

RIA. Class-specific RIAs for intact and trypsin-treated PV3 IgA, IgG, and IgM were carried out using the heavy chain–capture principle as described for IgM by Roivainen et al. [21]. In brief, 1:2 and 1:10 dilutions of the fecal suspension supernatants were added to duplicate wells on microtiter plates coated with monoclonal antibodies to human α, γ, and μ chains, respectively (gifts from Medix Biochemica, Kauniainen, Finland) and incubated for 2 h at 37°C. After washings with PBS containing 0.1% Tween 20...
(PBS-Tw20), ~30,000 cpm of 35S-Polio Sabin 3/well was added and was incubated for 1 h at 37°C. After washings with PBS-Tw20, scintillation liquid was added, and bound virus label was counted in a Wallac Microbeta Counter (Wallac Oy, Turku, Finland). The count minus background was used to indicate the relative antibody concentration. Specimens were scored antibody positive for a given immunoglobulin class if the mean cpm of the 4 wells exceeded the mean cpm of negative specimens plus 3 SD.

**Data analysis.** Frequencies of antibody responses against the 2 vaccines in the vaccine groups and the intensities of the responses were analyzed using the \( \chi^2 \) (with Yates correction) or Mann-Whitney test, respectively. A child was defined as a responder if the NT antibodies or a given RIA was positive in \( \geq 1 \) fecal specimen collected after the OPV challenge.

### Results

**Detection of poliovirus antibodies in vaccinees’ feces.** Fecal extracts of 66 children from the phase 3 clinical trial of the TryIPV vaccine studies were randomly chosen for testing for NT antibodies to intact and trypsin-treated PV3/Sabin in Vero cells. Thirty-six of these children belonged to the E-IPV group and 30 children to the TryIPV group. Children with \( \geq 1 \) post-challenge fecal specimen with detectable NT antibodies were considered responders in the analysis. More than 60% of the vaccinees in the E-IPV group developed measurable fecal NT antibodies during the follow-up (table 1). Only 2 children had measurable NT antibodies to both intact and trypsin-treated virus in the feces before the challenge. There were also 3 children in the E-IPV group in whom no virus excretion or antibody response was observed. The frequencies and the peak titers of the NT antibody responses in the E-IPV group in whom no virus excretion or antibody response were observed using the \( \chi^2 \) (with Yates correction) or Mann-Whitney test, respectively. A child was defined as a responder if the NT antibodies or a given RIA was positive in \( \geq 1 \) fecal specimen collected after the OPV challenge.

Fecal specimens of 31 children were randomly selected for testing for PV3-binding IgA, IgG, and IgM with class-specific RIAs. Sixteen of these children belonged to the E-IPV group, and 15 belonged to the TryIPV group. An IgA antibody response was most commonly found: >80% of the vaccinees in the E-IPV group developed an IgA response against the intact virus (table 2). One child had IgA antibodies in the feces before the challenge with both antigens, and another child as tested with the intact virus antigen. More frequent IgA responses against both virus preparations were seen in the E-IPV group, but the differences were not significant. Higher cpm values were observed in both groups with the intact virus antigen than with the trypsin-treated antigen (table 2), but, again, the differences were not statistically significant. Some IgG and IgM antibody responders were found, but the levels of these antibody responses were relatively low (table 2). About half of IgG and IgM responders were also IgA responders.

**Decline of virus excretion is associated with emergence of antibodies in feces.** Kinetics of antibody responses were analyzed separately for the 2 vaccine groups and against the 2 antigens. Once having appeared, the fecal NT antibody response usually, but not invariably, persisted through all the subsequent specimens. The measured antibody titers varied greatly, ranging from 1:2 to 1:512. Although both vaccine groups showed a proper intestinal response, the E-IPV group appeared to develop slightly better responses, as already described above. Therefore, in the following child-by-child analysis, we show the results from the E-IPV group only. In 70% of NT responders as tested with the intact virus antigen, and in 68% when with the trypsin-treated antigen, the appearance of the NT antibodies was associated with total ceasing of virus excretion (figure 1A). In the rest of the NT responders, virus excretion continued in spite of the NT response, but in most of them (86%) there was drop by \( \geq 1 \) log in the excreted virus titer at the time of appearance of the NT antibodies (figure 1B).

Children who developed an NT antibody response in the feces regularly also showed a coinciding PV3-binding IgA re-

### Table 2. Class-specific poliovirus antibody responses measured in fecal extracts by radioimmunoassay.

<table>
<thead>
<tr>
<th>Vaccine group (n), antigen</th>
<th>No. (%) of responders</th>
<th>Median (75th percentile) of peak cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgA</td>
<td>IgG</td>
</tr>
<tr>
<td>E-IPV PV3 (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>13 (8)</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Trypsin-cleaved</td>
<td>12 (75)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>TryIPV PV3 (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>9 (60)</td>
<td>5 (33)</td>
</tr>
<tr>
<td>Trypsin-cleaved</td>
<td>9 (60)</td>
<td>2 (13)</td>
</tr>
</tbody>
</table>

**NOTE.** Antigen, type of virus preparation used in assay; E-IPV, enhanced-potency inactivated poliovirus vaccine; n, number of children in group; PV3, poliovirus type 3; TryIPV, trypsin-modified E-IPV; responder, vaccinee with \( \geq 1 \) postchallenge fecal specimen positive in corresponding RIA. There was no significant difference in pairwise comparisons between vaccine groups or between antigen groups (\( \chi^2, P > .18 \)). “Peak cpm” refers to the highest bound cpm (out of input 30,000) recorded in any examined fecal specimen of a given vaccinee; median and upper 75th percentiles, respectively, are given for each group. There was no significant difference in pairwise comparisons between vaccine groups or between antigen groups (Mann-Whitney, \( P > .083 \)).
sponse. The relationship of the IgA response kinetics to virus excretion was similar to that of NT antibodies. In most IgA responders—69% with the intact antigen and 75% with the trypsin-treated antigen—a total disappearance of virus was seen at the time when IgA antibody became detectable. In 75% of cases where IgA excretion was overlapping with that of virus, there was a decrease by ≥1 log in the virus titer at the time when IgA first appeared (figure 1C). Similar coincidences of NT and IgA responses with decrease of virus excretion were also seen in the TryIPV group (data not shown).

Discussion

We analyzed in this study fecal antibody concentrations in 2 groups of IPV vaccinees after a challenge with a dose of monovalent OPV-3. No significant differences could be seen in the responses between the recipients of the regular E-IPV and children immunized with a TryIPV. These observations are in agreement with the earlier report that the challenge virus replicated in both groups with similar kinetics [18].

Several observations from previous studies suggest that an intestinal antibody response may have a role in intestinal resistance to reinfection by poliovirus [3, 4]. Relatively little is known about the factors that eliminate the primary infection from the body. Our current study supports the view that an intestinal antibody response, mainly consisting of IgA, may have a role here as well. A caveat in the interpretation is that the OPV recipients analyzed were not immunologically naive when challenged but had received 3 doses of IPV. A similar kinetic analysis with true primary vaccinees should be carried out to confirm the observations made here.

A large proportion, but not all, of the challenged children showed an antibody response measurable in fecal extracts. In some cases, the titers obtained were relatively high and were comparable to those measured in serum. IgA responses measured by an α-capture RIA coincided well with the NT responses. This suggests that the detected IgA is neutralizing. A formal proof for this could not be obtained, because repeated freezing and thawing of the extracts appeared to destroy the antibodies measurable by NT antibodies or RIA (data not shown). This is most likely due to the abundant proteases in stools [22], not completely inhibited by the FCS added into the extracts for this purpose. The fecal proteases have may be one reason for the failure to detect fecal antibodies in all vaccinees. They may also bring about inaccuracies in the measured antibody concentrations and thus provide a confounding factor in the interpretation of the results. In the current study, IgM and IgG class antibodies were also detected in some cases at low levels, as expected in mucosal secreta. The fecal proteases may also have affected the measurable IgG and IgM concentrations.

A fair coincidence was observed to exist between the decrease in the excreted challenge virus titer and the emergence of NT and IgA antibodies. The fact that the ceasing of PV excretion was not immediate in all individual cases is not surprising. It is not known how well fecal antibody concentrations reflect those at the virus replication sites.

In some children, a decrease in the excreted virus titer was followed by a “relapse” with increased virus titers, sometimes occurring relatively late during the 42-day observation period. It is possible that this phenomenon could be explained by emergence of mutant virus(es) resistant to the generated intestinal antibodies. Again, destruction of the antibodies during extended storage, including repeated freezing and thawing, prevented us from directly examining the matter. Antigenic mutants demonstrated by monoclonal antibodies are known to occur in OPV recipients [23].

In conclusion, we have shown that in recipients of a monovalent OPV type 3, excretion of the challenge virus showed a definite decrease at the time when intestinal NT antibodies, including IgA, become detectable. In most children, a complete ceasing of the virus excretion was found to follow the emergence of the antibody response. Several patterns of the response kinetics existed, however, and in some children the virus excretion also continued in spite of a remarkable antibody response. These results support the view that intestinal IgA response may be involved in the ceasing of a primary poliovirus excretion; however, the potential role of other significant factors, such as the cell-mediated immune response, cannot be excluded.

Acknowledgments

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References

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