Mucosal and Systemic Immunity against Poliovirus in Mice Transgenic for the Poliovirus Receptor: The Poliovirus Receptor Is Necessary for a Virus-Specific Mucosal IgA Response

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In view of the planned eradication of poliovirus, the suitability of transgenic mice bearing the human receptor for poliovirus (PVRtg mice) as a nonprimate animal model to study mucosal immunity against poliovirus was investigated. After intraperitoneal (ip) priming followed by ip or oral booster with live poliovirus, PVRtg mice had detectable IgA and IgG responses. The IgA response was restricted to PVRtg mice and could not be induced by oral immunization. After ip priming, PVRtg mice did shed virus in the stool, whereas control mice did not. Moreover, the amount of virus shed in the stools of PVRtg mice that had an IgA response after immunization was significantly lower than that of nonimmunized mice. A virus-specific mucosal IgA response is dependent on expression of the poliovirus receptor and is influenced by the route of immunization and the virus strain. PVRtg mice are a suitable model for the study of poliovirus-specific immunity and protection against poliovirus infection.

Polioymelitis is an acute paralytic disease caused by infection of the central nervous system by poliovirus, a member of the enterovirus genus of the family Picornaviridae. Poliovirus infection is initiated by oral ingestion of the virus, followed by binding to epithelial cells in the tonsils and M cells in the intestine. After local replication, the virus is transmitted into cervical and mesenteric lymph nodes and then into the circulation. When viremia is established, the virus invades the central nervous system, and paralytic poliomyelitis may occur as a result of destruction of motoneurons [1].

A dramatic reduction in the incidence of poliomyelitis was achieved by vaccination with either attenuated live oral poliovirus (OPV) or wild-type inactivated poliovirus (IPV) [2]. Although both vaccines protect persons from illness by induction of circulating neutralizing antibodies, OPV is a more potent inducer of mucosal antibodies than is IPV [3–6]. Mucosal immunity is becoming increasingly important in the advancing steps of the World Health Organization’s polio eradication campaign [7]; the chain of transmission of poliovirus needs to be interrupted to free the world from poliovirus. Therefore, it is essential to know which vaccination strategy will induce an intestinal immune response that prevents replication and shedding of the virus, especially in the final stages of the eradication program. Locally produced secretory IgA is the predominant immunoglobulin isotype along mucosal surfaces and is associated with reduced virus excretion after OPV challenge [6]. To elicit a mucosal IgA response in the intestine, antigens are transported across the epithelium by M cells and are subsequently processed and presented to B lymphocytes in the Peyer’s patches, which are precursors of IgA-producing plasma cells in the gut [8].

Humans and monkeys are the only natural hosts for poliovirus, because their cells express a surface molecule that acts as a poliovirus receptor [1, 9]. However, transgenic mice bearing the human receptor for poliovirus (PVRtg) have been developed; they are susceptible to all 3 poliovirus serotypes and have been used for neurovirulence studies [10–13]. Although some studies have addressed systemic immunity against poliovirus [14], it is unknown whether these mice are suitable to use in studying poliovirus-specific mucosal immunity. The aim of the present study was to investigate whether PVRtg mice are able to elicit a mucosal IgA response against poliovirus serotype 1. We studied the route of immunization and the influence of the virus strain used for immunization with respect to their ability to induce antibodies at the mucosal site and to protect from reinfection.

Materials and Methods

Animals. Homozygous transgenic mice carrying the human PVR gene (ICR-PVRtg21), generated as described elsewhere [11],

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Guidelines for animal experimental work were followed in this study. The study design was approved by the National Institute of Public Health and the Environment Ethical Committee for Animal Experiments. Financial support: Princes Beatrix Fonds, The Netherlands.

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were obtained from A. Nomoto (University of Tokyo). They were bred and housed in an isolator cage according to World Health Organization guidelines [15]. Kidney sections of the PVRtg mice were tested for the presence of the PVR gene by polymerase chain reaction [16]. Nontransgenic ICR mice (Harlan France SARL, Gannat, France) were used as controls.

**Virus**. The virulent Mahoney strain and attenuated Sabin strains of poliovirus serotype 1 were used in this study. Poliovirus-infected cell lysates were purified by use of a CsCl gradient in PBS. Subsequently, the titer of infectious virus was determined by virus titration assays on HEp-2 cells [17].

The presence of poliovirus was confirmed by microneutralization with serotype 1-specific rabbit antisera. Procedures [17]. The optimal dilution of the conjugates had been determined by checkerboard titrations. After 4 washes with PBS-Tw, the IgA plates were incubated with alkaline phosphatase-conjugated streptavidin (dilution, 1:2000) for 1 h at 37°C and then washed 6 times with PBS-Tw. Substrate solution (100 μL of 1 mM 5-bromo-4-chloro-3-indolyl phosphate in 2-amino-2-methyl-1-propanol, adjusted to pH 10.25 with concentrated HCl) in 0.6% agarose was added to the wells, and the plates were incubated for 2 h at 37°C. Plaques appearing as blue spots were counted.

**ELISpot assays**. Wells of ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 1.2 μg/mL of anti-poliovirus type 1 serum, blocked with 1% bovine serum albumin in PBS-Tw, incubated with D antigen in PBS-Tw containing 0.5% bovine serum albumin, and washed as described for the ELISpot assay. After washing, serum samples (dilution, 1:50) and intestinal washings, intestinal scrapings, and saliva (dilutions, 1:4) in PBS-Tw (containing 0.05 M NaCl and 5% gelatin hydrolysate [Boehringer] for dilution of the intestinal secretions) were incubated for 1 h at 37°C. After 4 washes with PBS-Tw, the plates were incubated with biotin-labeled goat anti-mouse IgA (α-chain specific; Southern Biotechnology Associates, Birmingham, AL; dilution, 1:8000) and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma; dilution, 1:2000) was added to the wells and incubated for 1 h at 37°C. The optimal dilution of the conjugates had been determined by checkerboard titrations. After 4 washes with PBS-Tw, the IgA plates were incubated with alkaline phosphatase-conjugated streptavidin (dilution, 1:2000) for 1 h at 37°C and then washed 6 times with PBS-Tw. Substrate solution (100 μL of 1 mM 3-bromo-4-chloro-3-indolyl phosphate in 2-amino-2-methyl-1-propanol, adjusted to pH 10.25 with concentrated HCl) in 0.6% agarose was added to the wells, and the plates were incubated for 2 h at 37°C. Plaques appearing as blue spots were counted.

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itive when the OD was above the cutoff levels (mean + 3 SD of the samples of nonimmunized mice).

Statistical methods. Antibody responses between PVRtg mice and control mice were tested for differences by Student’s t test.

Results

Ip immunization with poliovirus induces a systemic but also a mucosal antibody response in PVRtg mice. To examine whether PVRtg mice developed a poliovirus-specific humoral antibody response, mice were immunized ip with Sabin poliovirus serotype 1. Parallel groups of mice were mock-immunized by ip injection with PBS. The induction of poliovirus-specific antibody-secreting cells in the spleens of PVRtg and control mice was analyzed by ELISpot assay, and poliovirus-specific IgG and IgA in serum, intestinal washings, and intestinal scrapings were determined by ELISA. Two weeks after ip priming, IgG antibodies were found in serum of PVRtg mice (figure 1A), and 15 ± 17 IgG-secreting cells/10^6 cells were found in the spleens of these mice (figure 1B), whereas no IgG or antibody-secreting cells were found in serum and spleens of control mice. Subsequently, 2 weeks after priming, mice received an ip booster immunization with 10^7 TCID50 of Sabin virus. Two weeks after the booster, both PVRtg and control mice produced a high amount of IgG in the serum (figure 1A) and had 20–30 IgG-secreting cells/10^6 cells in the spleen (figure 1B). Mock-immunized PVRtg mice and control mice did not show any poliovirus-specific IgG response (figure 1A, 1B).

After ip priming with Sabin virus, neither PVRtg mice nor control mice had IgA in their intestinal secretions. However, after the ip booster immunization, PVRtg mice generated an IgA response in both intestinal washings and scrapings, whereas control mice did not produce intestinal IgA (figure 1C, 1D). No IgA-secreting cells were observed in the spleens, mesenteric lymph nodes, or Peyer’s patches of the PVRtg mice or control mice (data not shown). These results showed that the poliovirus receptor is necessary for the induction of an intestinal IgA response but not for a systemic IgG response.

The route of immunization is important for induction of a poliovirus-specific IgG or IgA response in PVRtg mice. The result of ip priming and boosting with poliovirus in PVRtg mice was a clear mucosal IgA response, but this is not the normal route of immunization with OPV or of wild-type infection. Therefore, we investigated whether oral immunization would also be able to induce an IgA response. Oral and ip priming were compared, and both immunizations were followed by an oral booster at week 4 after priming. The induction of IgG and IgA was measured at week 4 after the oral booster immunization. Priming

Figure 1. IgG present in serum (A), IgG-secreting cells in spleen (B), IgA in intestinal washings (C), and IgA in intestinal scrapings (D) of transgenic mice bearing the human poliovirus receptor (PVRtg) and of control mice measured 2 weeks after intraperitoneal (ip) priming and 2 weeks after ip booster immunization. Mice were primed with 10^7 TCID50 of Sabin poliovirus type 1 or PBS and were boosted (+) or not boosted (−) 2 weeks later with 10^7 TCID50 of Sabin poliovirus type 1 or PBS. Data are optical density (OD) at 405 nm (except for B, IgG-producing cells/10^6 cells) and represent mean ± SD of values of 4 individual mice.
ip with $10^5$ TCID$_{50}$ of Sabin virus in combination with an oral booster of $10^8$ TCID$_{50}$ of Sabin virus resulted in the presence of IgA antibodies in intestinal washings and scrapings (figure 2A, top and middle) and a low level of IgA (figure 2A, bottom) in the serum of PVRtg mice. This immunization scheme also induced IgG in serum and intestinal secretions of PVRtg mice (figure 2B). In contrast to the PVRtg mice, control mice did not generate any IgG or IgA antibodies in serum or intestinal secretions after ip priming in combination with oral booster immunization with Sabin virus (figure 2). However, oral priming alone or in combination with a booster of $10^8$ TCID$_{50}$ of Sabin virus did not generate any IgA in intestinal washing, intestinal scrapings, or serum (figure 2A) or induce IgG antibodies in serum (figure 2B, bottom) or spleens (data not shown) of PVRtg mice or control mice. Moreover, oral immunization with $10^8$ TCID$_{50}$ of Mahoney virus followed by 3 oral booster immunizations with $10^8$ TCID$_{50}$ of Mahoney virus also did not induce any IgA or IgG response in the intestinal secretions of the PVRtg mice or control mice (data not shown). These results indicate that ip priming of PVRtg mice with poliovirus is essential for the induction of a virus-specific intestinal IgA response and a systemic IgG response.

Influence of the virus strain on the induction of mucosal IgA and systemic IgG: comparison of immunization with Mahoney

**Figure 2.** IgA (A) and IgG (B) present in intestinal washings (top), intestinal scrapings (middle), and serum (bottom) of transgenic mice bearing human poliovirus receptor (PVRtg) and control mice, measured 4 weeks after oral priming with $10^8$ TCID$_{50}$ of Sabin poliovirus or intraperitoneal (ip) priming with $10^5$ TCID$_{50}$ of Sabin virus and 4 weeks after oral booster immunization with $10^8$ TCID$_{50}$ of Sabin virus. Four weeks after priming, mice received oral booster. Data are optical density (OD) at 405 nm and represent mean ± SD of values of 4 individual mice.
virus and immunization with a Sabin strain. In combination with an oral booster, ip priming immunization with Sabin poliovirus resulted in a clear mucosal IgA and a systemic IgG response in PVRtg mice. However, because we are interested in the IgA response after vaccination with OPV virus and after infection with poliovirus, the immune responses after immunization with attenuated Sabin poliovirus and with Mahoney virus were compared. Because Mahoney virus is much more virulent than Sabin virus, a 10-fold lower dose was used for the immunizations. Mice were primed ip with $10^5$ TCID$_{50}$ of the Mahoney strain or $10^6$ TCID$_{50}$ of Sabin virus serotype 1, which was followed by an oral booster immunization with $10^7$ TCID$_{50}$ of Mahoney or $10^8$ TCID$_{50}$ of Sabin poliovirus, respectively. Subsequently, the presence of poliovirus-specific IgA in the intestinal secretions and saliva of PVRtg mice and control mice was determined at weeks 2 and 4 after the oral booster immunization (figure 3). Already at week 2 after the oral booster immunization with Mahoney virus, the PVRtg mice had a significant amount of IgA in both intestinal washings and scrapings, compared with control mice (figure 3A, top and middle). Similarly, at week 4 after the booster immunization with Mahoney virus, the intestinal IgA response and the amount of salivary IgA were significantly higher than those in the control mice (figure 3A). In contrast, at week 2 after the booster immunization with Sabin virus, no IgA was found in any of the mucosal secretions tested (figure 3B). However, at week 4 after the oral booster immunization with Sabin virus, the PVRtg mice produced IgA in intestinal washings, intestinal

**Figure 3.** IgA response in intestinal washings, intestinal scrapings, and saliva of transgenic mice bearing human poliovirus receptor (PVRtg) mice and control mice (con) after intraperitoneal priming and oral booster with wild-type Mahoney poliovirus (A) or Sabin serotype 1 poliovirus (B). Mice were primed with $10^5$ TCID$_{50}$ of Mahoney virus or $10^6$ TCID$_{50}$ of Sabin virus and received booster with $10^7$ TCID$_{50}$ of Mahoney or $10^8$ TCID$_{50}$ of Sabin virus, respectively, 4 weeks after priming. Data are optical density (OD) at 405 nm and represent mean ± SD of values of 4–6 individual mice at weeks 2 and 4 after booster. *Significant difference ($P < .05$) between PVRtg mice and control mice.
The poliovirus receptor is necessary for a virus-specific mucosal IgA response. Because the number of animals in each experimental group was low, results from all experiments of ip priming followed by an oral booster immunization were lumped for statistical analysis (table 1). When mice were immunized with poliovirus ip in combination with an oral booster, 96.2% of the PVRtg mice immunized with Mahoney virus generated an intestinal IgA response, whereas none of the control mice showed a mucosal IgA response (table 1). After immunization with the Sabin strain according to the same immunization schedule, almost half the PVRtg mice generated an intestinal IgA response at week 4 after the booster immunization, whereas none of the control mice produced IgA at the mucosal surfaces (table 1). These results clearly demonstrate that the poliovirus receptor is necessary for the induction of a virus-specific mucosal IgA response and that wild-type Mahoney virus induces a mucosal immune response better than does Sabin virus.

PVRtg mice shed virus in the stool after ip inoculation with wild-type poliovirus, which is greatly reduced by the presence of a mucosal immune response in these mice. After ip inoculation of PVRtg mice with 10^5 TCID_{50} of Mahoney virus, virus was excreted in the feces of these mice. The amount of virus present in the stool samples of PVRtg mice was high from day 2 until day 6 and gradually decreased on days 7 and 8 (figure 4). Five of the 20 PVRtg mice showed paralysis and died by day 6 after ip inoculation with Mahoney virus. A virus titer (mean ± SD) of 1 ± 0.6 × 10^5 TCID_{50} was found in the brain tissue of these mice.

After ip priming with 10^5 TCID_{50} of Sabin virus, a virus titer of 10^2 TCID_{50} of 100 mg of feces was shed in the feces of PVRtg mice at days 3 and 4 and not in that of the controls. None of the PVRtg mice died or showed paralysis.

We determined whether a mucosal immune response could limit the virus shedding in the feces of PVRtg mice. Mice immunized ip in combination with an oral booster immunization with Mahoney virus were challenged ip with 10^5 TCID_{50} of Mahoney virus at week 4 after the booster immunization. At the time of challenge, after immunization, a clear IgA response was present in the intestine (OD at 405 nm of 1.8 ± 0.5, mean ± SD); also, an IgG response (2.0 ± 0.4) was found in the intestines of the PVRtg mice. The immunized PVRtg mice, however, excreted a very low amount of virus in the stool compared with that excreted by nonimmunized PVRtg mice, as described above (figure 4). None of the immunized mice died after challenge with Mahoney virus. Control mice, regardless of whether immunized, excreted only a very low amount of poliovirus in the stool after ip challenge with Mahoney virus (figure 4). From these results, we conclude that the IgA and IgG antibodies produced in the intestines of PVRtg mice after
immunization are associated with protection against virus excretion after challenge with wild-type virus.

Discussion

When inoculated intracerebrally, PVRtg mice show clinical symptoms similar to those observed in humans and monkeys, which has led to their use for poliovirus neurovirulence tests [10, 12]. However, little is known about poliovirus-specific antibody responses in these mice and whether they are suitable for study of mucosal immunity. In humans, vaccination with OPV and IPV elicits a strong systemic IgG response that is even more elevated after natural exposure to wild-type poliovirus [5]. Similarly, parenteral immunizations are able to induce virus-specific systemic IgG responses in mice [14]. To our knowledge, studies of mucosal immunity in PVRtg mice have not been reported. In fact, it has been suggested that these mice cannot be used for mucosal infection [12, 21]. Thus far, attempts to demonstrate viral replication after oral inoculation in mice expressing high levels of human poliovirus receptor by M cells and enterocytes have failed [22], which may be attributed to a blockade independent of receptor binding. This is supported by the finding that many tissues of PVRtg mice remain refractory to poliovirus infection, despite expression of poliovirus receptor RNA [23]. As a result, studies of mucosal immunity after oral inoculation are likely to fail. Similarly, although induction of IgA antibodies is thought to require the direct contact of antigen with the mucosal surfaces [24–26], oral immunizations with poliovirus in our PVRtg mice indeed failed to induce any IgA, confirming previous observations [21]. Although poliovirus is able to enter M cells apically in the human intestine [27], this route likely is of minor importance in PVRtg mice. It remains to be seen whether basolateral entry takes place.

In the present study, however, we adapted the PVRtg mouse model to study virus-specific mucosal IgA and systemic IgG responses, by use of an alternative route of infection, thus circumventing the intestinal route. Although the absence of local replication of poliovirus suggested that PVRtg mice are not suitable for studying mucosal immunity, we observed a clear IgA response in both the intestine and the saliva of the PVRtg mice, when ip immunization with poliovirus was followed by an ip or oral booster. Unexpectedly, the ip immunization was followed by virus shedding in the stools of PVRtg mice, thereby enabling us to use this route for challenge experiments. Therefore, although this route of immunization is somewhat artificial, these mice provide us with a useful animal model for study of the poliovirus-specific mucosal immune response. Moreover, induction of paralysis and death of some of the PVRtg mice occurred after ip infection with wild-type poliovirus. The presence of virus in the brains of some mice after ip infection with Mahoney virus and the observed fecal shedding of the virus imply that replication of the virus occurs in PVRtg mice, which leads to viremia, as has been described for monkeys after oral inoculation of poliovirus [1].

The mechanisms explaining the induction of IgA after ip immunization in PVRtg mice are not yet clear. Because poliovirus is able to replicate in human monocytes [28], it is conceivable that after ip inoculation of poliovirus in PVRtg mice, the virus will enter peritoneal macrophages and may also replicate in these cells. These cells are able to migrate to the Peyer’s patches, where B cells could be primed for the production of IgA [29]. We have some preliminary results indicating that poliovirus replicates in the mesenteric lymph nodes and Peyer’s patches of PVRtg mice after ip inoculation of poliovirus (authors’ unpublished data). Another possibility is the involvement of B-1 cells in the peritoneal cavity, which could be activated after ip immunization in PVRtg mice and would migrate into the lamina propria, where they produce IgA [30–33].

By use of the PVRtg mouse model, we found a local immune response and shedding of the virus in the feces, which is strongly reduced after renewed contact with the virus. Whereas the mechanisms of protection are not yet known in these mice, it is likely that mucosal IgA antibodies play a critical role by preventing attachment of the virus to cells of the Peyer’s patches [8, 34] or by forming complexes with the virus intracellularly, inhibiting replication of the virus at the mucosal surface [35]. This is also suggested by Fiore et al. [36], who found poliovirus-specific neutralizing IgA in mice. However, the role of T cells needs to be addressed in future studies.

Although PVRtg mice and control mice clearly differ in local mucosal immune responses against poliovirus, systemic IgG responses are seen in both PVRtg and control mice, albeit at slightly different levels. Differences in antigen processing and presentation between PVRtg and control mice might explain the differences in the level of antibody responses between these groups of mice [37, 38]. However, the most likely explanation is the different amounts of antigen present in the 2 groups of mice, because of differences in viral replication, after immunization with poliovirus.

Besides systemic IgG, we also observed a large quantity of poliovirus-specific IgG locally in the intestines of PVRtg mice. This is not likely due to cross-reactivity between the IgA and IgG ELISAs, because serum samples of some PVRtg mice showed a high IgG response but a low level of IgA. And, conversely, some saliva samples were strongly positive for IgA but not for IgG. Virus-specific IgG present in the intestines of mice has also been described by others [39]. These IgG antibodies could be partly produced locally by plasma cells, because 5%–15% of these cells in the human mucosal tissues produce IgG [40]. However, the majority of IgG present at the mucosal surface has been assumed to be derived from the serum through passive transudation down a concentration gradient [41]. This suggests that IgG may contribute to mucosal protection through intracellular association with antigen and subsequent activation of complement. It is conceivable that local IgG plays
a role in the protection against poliovirus infection after vaccination with IPV [42]. This will be tested in future experiments.

In conclusion, we demonstrated that PVRtg mice are useful for the study of the mucosal immune response against poliovirus, because they develop a local immune response and shed virus in the feces. Moreover, this immune response protects PVRtg mice against ip infection with wild-type poliovirus, so we expect the PVRtg mouse model to be useful for further challenge experiments after immunizations with OPV and IPV. The mechanisms explaining the induction of the poliovirus-specific mucosal immune response in PVRtg mice will be the subject of future studies.

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