Poliomyelitis in Intraspinally Inoculated Poliovirus Receptor Transgenic Mice

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Received February 20, 1998; returned to author for revision April 29, 1998; accepted December 11, 1998

INTRODUCTION

Poliovirus is a small positive-stranded RNA virus with a tropism for the human nervous system. When neuroinvasive, this picornavirus causes a central nervous system (CNS) disease known as poliomyelitis. Natural transmission of poliovirus occurs via a fecal to oral route. Once ingested, poliovirus replicates first in the oropharynx and then in the gastrointestinal tract prior to establishing viremia (Sabin, 1956). In a small percentage of cases, poliovirus is transported to the CNS, either through the blood–brain barrier (Koike et al., 1990), is a member of the immunoglobulin superfamily (Mendelsohn et al., 1989). A homolog of this gene is also encoded by nonhuman primates, causing them to be susceptible to poliovirus infection and disease only when virus is inoculated into the CNS (Koike et al., 1992). Mice transgenic with the PVR gene (TgPVR mice) may, therefore, be useful in exploring poliovirus replication and subsequent disease pathogenesis.

In this report, TGM-PRG-1 and TGM-PRG-3 (Deatly et al., 1998) mice were inoculated in the spinal cord with modified live polioviruses to examine poliovirus neurovirulence and poliomyelitis. This study focuses on the observations of the clinical signs of poliomyelitis in infected mice (similar to those of human disease) which become paralyzed, die, or are unaffected. The results of six intraspinal (IS) challenge studies demonstrated the following: a higher proportion of mice were affected and the clinical signs of poliomyelitis were more severe when they were inoculated with a neurovirulent poliovirus (vaccine 3B) compared to an attenuated poliovirus reference strain (i.e., WHO/III, used as a passed vaccine control in the monkey neurovirulence test); the proportion of mice affected and disease severity increased with increasing dose of poliovirus; and a higher proportion of male mice were

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affected than female mice when inoculated with vaccine 3B or the WHO/III reference virus.

By intracranial (IC) inoculation, the two TgPVR mouse lines appear to have similar susceptibilities to wild-type III poliovirus, even though their receptor expression levels differ by threefold (Deatly et al., 1998). The same two mouse lines, TGM-PRG-1 and TGM-PRG-3, were also used in this study, and the results obtained for each mouse line were compared to assess their relative susceptibilities to less neurovirulent type III polioviruses administered IC. As in the previous study, these two mouse lines were similar in their susceptibility to poliovirus, even when less neurovirulent virus strains were inoculated in the spinal cord. Therefore, the dosage of the PVR gene and the difference in abundance of the receptor in the brain (Deatly et al., 1998) do not affect the overall susceptibility of TgPVR mice to type III polioviruses inoculated in the CNS tissues. However, receptor gene dosage and abundance of pvr in CNS tissues affect the time to onset of disease. By statistical analysis, the difference in failure time of these two mouse lines was significant. The failure time of TGM-PRG-3 mice, the mouse line with a threefold higher level of pvr in the brain, is at least 1 day shorter than that of TGM-PRG-1 mice. Because in the majority of cases, clinical signs in mice develop from days 2 to 5, 1 day represents a 25% shorter failure time for TGM-PRG-3 mice relative to TGM-PRG-1 mice.

RESULTS

IS inoculation of TGM-PRG-1 and TGM-PRG-3 mice with polioviruses

Factors affecting poliovirus neurovirulence. To investigate how TGM-PRG-1 and TGM-PRG-3 mice respond to different strains of type III poliovirus, mice from both transgenic lines were inoculated with either a neurovirulent type III poliovirus, vaccine 3B, or an attenuated poliovirus type III reference strain, WHO/III. These polioviruses differ in the percentage content of 472-C, which correlates with neurovirulence of type III polioviruses (Chumakov et al., 1991). We determined that vaccine 3B contains 4.78% C and the WHO/III reference virus contains 0.44% C at position 472, which correlates with neurovirulence of type III polioviruses (Chumakov et al., 1991). We determined that vaccine 3B contains 4.78% C and the WHO/III reference virus contains 0.44% C at position 472, which is similar to results reported by others (Dragunsny et al., 1996). Wild-type III poliovirus has approximately 100% C at position 472.

Groups of 10 mice from each line were inoculated with each of four doses (10^2, 10^3, 10^4, and 10^5 TCID_{50}) by the IS route. The virus inoculum was delivered to the spinal cord to target motor neurons enervating the hind limb muscles. Following challenge, the mice were observed for 14 days for the development of hind limb paralysis and/or death. Any mice without signs of poliomyelitis were designated “unaffected.” The parameters of neurovirulence expressed by TgPVR mice following virus challenge, i.e. paralysis and mortality, are similar to clinical signs of spinal poliomyelitis in humans.

Poliovirus strain. The proportions of TGM-PRG-1 and TGM-PRG-3 mice inoculated with vaccine 3B or WHO/III reference virus showing clinical signs (paralysis or mortality) for each of the six tests are illustrated in Table I. Even though there is variability from test to test in the proportions of mice affected at the respective doses, the mean proportions demonstrate that higher numbers of TGM-PRG-1 and TGM-PRG-3 mice developed paralysis and/or died when inoculated with vaccine 3B than with

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\(^a\) Clinical signs include paralysis and/or death.

\(^b\) The doses are in log_{10} TCID\(_{50}\).
the WHO/III reference virus at comparable doses. In addition, clinical signs of poliomyelitis were more severe in mice inoculated with vaccine 3B than the WHO/III reference; i.e., a greater proportion of the mice that developed clinical signs died.

Since the proportions of clinical signs were highly similar for the two mouse lines, data from both were compiled to determine the effects of virus strain on response frequencies. Figure 1 displays logistic regression curves for paralysis of the 959 TGM-PRG-1 and TGM-PRG-3 mice inoculated IS with the type III poliovirus vaccines, WHO/III and vaccine 3B. To illustrate the difference in the probability of paralysis for each poliovirus tested at log10 dose of 4.0 TCID50/mL, TgPVR mice have a 60–62% probability to be affected by vaccine 3B and a 28–30% probability to be affected by WHO/III. The dose at which 50% of the mice were paralyzed (PD50) is 10^{2.5} TCID50 for vaccine 3B and 10^{4.6} TCID50 for the WHO/III reference virus. Therefore, significantly less vaccine 3B virus is needed to cause paralysis than is required for the reference virus. Also, both TGM-PRG-1 and TGM-PRG-3 mice are able to differentiate the neurovirulent poliovirus from the reference strain on the basis of clinical signs.

Poliavirus dose. Table I also illustrates the dose-dependent response of these poliovirus-sensitive mice to each poliovirus. The higher the dose of virus inoculated, the greater the number of mice that developed clinical signs of poliomyelitis. The mean PD50 values of all six experiments for each poliovirus and each mouse line were calculated (Table I). As illustrated, the six tests showed similar PD50 values for each virus in either mouse line. TGM-PRG-1 and TGM-PRG-3 mice had 0.85 and 1.00 log lower PD50 values, respectively, for the vaccine 3B than the WHO/III reference challenges.

Comparing poliovirus susceptibility of TGM-PRG-1 and TGM-PRG-3 mouse lines

Logistic regression was used to compare the relative susceptibilities of TGM-PRG-1 and TGM-PRG-3 mice to poliovirus infection. Figure 1 portrays the dose responses of TGM-PRG-1 and TGM-PRG-3 mice inoculated with vaccines 3B and WHO/III. As depicted, both mouse lines showed similar response curves to each of the viruses. Less than 0.3 log dose units separate TGM-PRG-1 and TGM-PRG-3 regression curves for vaccine 3B at the 50% probability level, and the two lines are virtually identical for the WHO/III virus.

Contingency table analysis of the six tests combined found no significant differences between TGM-PRG-1 and TGM-PRG-3 mice in paralysis and mortality rates (P values of .1432 and .2051, respectively). Taken together, these results demonstrate that TGM-PRG-1 and TGM-PRG-3 mice are very similar in their susceptibilities to type III polioviruses.

Failure time, the time from inoculation of the virus to onset of disease signs, was compared by proportional hazards to evaluate potential differences in poliovirus susceptibility of the two mouse lines. For these type III viruses, the peak time for development of clinical signs is from day 2 to day 5 postinoculation. Failure times for lines TGM-PRG-1 and TGM-PRG-3 are illustrated in the survival curves shown in Fig. 2. Clinical signs appeared more rapidly in TGM-PRG-3 mice. Adjusting for experiment, dose, and sex in the regression, the P value for the difference between the two mouse lines was significant (P = .0384).
Effect of gender on development of poliomyelitis in TgPVR mice

Figure 3 illustrates the differences in responses of male and female TGM-PRG-1 and TGM-PRG-3 mice to a challenge with vaccines 3B and WHO/III for all six tests. The probability of paralysis for males and females from both mouse lines post-IS inoculation with the WHO/III reference virus is plotted versus the dose (Fig. 3A). The PD_{50} values for females and males inoculated with the WHO/III reference virus were 4.93 and 4.10, respectively. Because higher numbers of male mice develop clinical signs of poliomyelitis than female mice, male mice are more susceptible to poliovirus infection. The response curves of males and females, which differ from each other, were nearly identical for both mouse lines (data not shown).

To determine if receptor dosage plays a role in the differential poliovirus susceptibility of male and female mice, pvr abundances in CNS tissues of male and female TGM-PRG-3 mice were compared by Western blot analysis. As illustrated in Fig. 4, the level of pvr in the brain, brain stem, and spinal cord tissues was similar in TGM-PRG-3 males and females. Similar pvr abundances were also observed in TGM-PRG-1 male and female CNS tissues (data not shown). Abundance of pvr, therefore, is not responsible for the increased poliovirus sensitivity of male mice.

DISCUSSION

The use of TgPVR mice has enabled the identification of different parameters that contribute to the disease state following poliovirus infection, including the strain of poliovirus, the dose of virus, and the gender of the mouse. The relative neurovirulence of the strain of poliovirus, as defined by the monkey neurovirulence test for OPV vaccines, is a major contributor to disease severity in TgPVR mice. This shows that TgPVR mice respond to various polioviruses similarly to humans and monkeys. Accumulated data from these six poliovirus mouse challenge tests clearly demonstrate repeatability of poliovirus strain and dose differentiation by these two mouse lines. The strong dose dependence exhibited by TgPVR mice, however, is not observed in monkeys (Furesz and Contreras, 1993).

These results testify that TgPVR mice are able to orchestrate the stages of a poliovirus infection beyond virus binding and cell entry when poliovirus is delivered to the CNS. Host factors, necessary for translation and replication of the poliovirus genome (Barton et al., 1995; Gamarnik and Andino, 1996), must be present in mouse CNS cells infected by poliovirus. Further support for the presence of these host factors in the mouse CNS is the similarity of the neuropathology in poliovirus-infected TgPVR mice, monkeys, and humans (Brownell and Tomlinson, 1984; Ren et al., 1990; Deatly et al., unpublished results).

Perhaps more interesting, and less well understood, is the strong contribution of gender to poliovirus neurovirulence in these transgenic mice. As illustrated, male transgenic mice are more likely to develop poliomyelitis than female mice and a higher proportion of males than
females die after developing paralysis. Epidemiology studies in humans suggest the same phenomenon; i.e., human males are more susceptible than females to enteroviruses, especially polioviruses (with a male:female ratio between 1.5:1 and 2.5:1, respectively). These ratios indicate that 60–70% of all enteroviral disease occurs in males (Moore, 1982; Morens and Pallansch, 1995). While the biological significance of this observation is not known in humans, Gelfand (1962) correlates these observations with extended periods of virus excretion, higher excretion titer, and higher infection rate in otherwise healthy male versus female subjects. The overall ratios of poliomyelitis in mice over the four doses is 1.9:1 (males to females), a ratio similar to that in humans. Whereas it is not yet known if the excretion times are longer and titers are higher in male versus female TgPVR mice, it is known that the incidence of disease is higher in males.

The relative abundance of pvr was compared in CNS tissues of male and female mice to gain insight into the differential susceptibility based on gender. However, the abundance of the poliovirus receptor is similar in the male and female CNS tissues in both transgenic mouse lines. Perhaps a difference in expression of another gene in male versus female neurons (Amrein and Axel, 1997), potential differences in immune responses between males and females, and/or hormonal differences affect susceptibility to poliovirus infections (Shanahan, 1997). Others have speculated that the higher proportion of disease in adult human males versus females is attributable to muscle trauma or increased muscular activity (Moore, 1982; Morens et al., 1991) or injury-provoked poliomyelitis (Gromeier and Wimmer, 1998). While the biological significance of this phenomenon is intriguing, the cause of increased poliovirus susceptibility in males may be difficult to determine.

Interestingly, even though the poliovirus receptor is threefold more abundant in the brains of TGM-PRG-3 than in those of TGM-PRG-1 mice (Deatly et al., 1998), there is no statistically significant difference in poliovirus susceptibility between these mouse lines. This result, first observed in TgPVR mice inoculated IC with wild-type III (Leon) poliovirus into mice from both mouse lines (Deatly et al., 1998), is confirmed here with less neurovirulent polioviruses inoculated into the spinal cord. IS, rather than IC inoculation, was used for the experiments in this report to ensure consistency of delivery of poliovirus to the same target site. This is possible in the spinal cord because the transverse processes highlight specific vertebrae. IS inoculation data demonstrate that TGM-PRG-1 and TGM-PRG-3 mice are equally susceptible to poliovirus infection; both capable of distinguishing differences in neurovirulence for different poliovirus strains/vaccines; and equivalent in the frequency and magnitude of clinical signs produced in response to virulent virus challenge. In contrast, two poliovirus-sensitive transgenic mouse lines produced in the ICR genetic background, TgPVR1 and TgPVR21 (a ratio of 10:3, respectively), differ in poliovirus susceptibility which appears to correlate with poliovirus receptor and gene dosage (Koike et al., 1994). The reason(s) for the difference in these results is not yet understood.

Increased receptor dosage does, however, appear to affect the time to onset of disease in TGM-PRG-1 and TGM-PRG-3 mice. Failure time is significantly shorter in TGM-PRG-3 mice relative to TGM-PRG-1 mice.

It is clear, from these and other studies, that the receptor plays a major role in the initial interaction of the virus with CNS tissues, since nontransgenic mice do not develop poliomyelitis (Ren et al., 1990; Deatly et al., unpublished results). Future experiments are planned to determine if the poliovirus receptor is functional in facil-
mitating an infection if the virus is administered orally. These studies should be key to elucidating the pathway for poliovirus infections affecting CNS tissues via the natural route of infection.

Since poliovirus-infected TgPVR mice develop clinical signs of poliovirus similar to those of humans and they differentiate the neuropatholence of various strains according to dose, TgPVR mouse is an appropriate animal model for the safety testing of oral poliovirus vaccines. Results from these experiments and others (Dragunsky et al., 1996) also suggest the possibility of using clinical signs of paralysis and mortality as the assay endpoint for this regulatory test, rather than neuropathological evaluation of CNS tissues.

MATERIALS AND METHODS

Vaccines

Vaccine 3B and WHO/III polioviruses were obtained from Dr. David J. Wood (National Institute of Biological Safety and Standards, United Kingdom). The WHO/III (a Sabin original after two passages—SO + 2) poliovirus (an attenuated vaccine based upon the monkey neurovirulence test; Dragunsky et al., 1996) is used as the reference virus for this assay. All oral poliovirus vaccines must be subjected to the monkey neurovirulence test to demonstrate consistency and safety of vaccine manufacture. Vaccine 3B poliovirus failed the monkey neurovirulence test.

Mutation analysis by PCR restriction enzyme cleavage (MAPREC) assays (Chumakov et al., 1991) were performed on these polioviruses to determine the percentage of cytosine (C) at nucleotide 472. The change from a uracil (U) to a C at nucleotide 472 in type III OPV vaccines correlates with an increase in histologic lesion scores in monkeys inoculated in the spinal cord (Chumakov et al., 1991). Vaccines which fail the monkey neurovirulence test generally have greater than 1% 472-C (Dragunsky et al., 1996).

Maintenance of mouse lines

TGM-PRG-1 and TGM-PRG-3 are new mouse lines transgenic with the human poliovirus receptor gene and are recently characterized (Deatly et al., 1998). TGM-PRG-1, TGM-PRG-3, and nontransgenic mice are maintained and bred under viral antigen-free conditions at Charles River Laboratories. Barrier-derived homozygous TGM-PRG-1 and TGM-PRG-3 male mice were bred to C57BL6/J (nontransgenic) females to produce offspring hemizygous for the transgene. Maintenance and transport of these transgenic mouse lines were performed in compliance with the guidelines recommended by the WHO in a memorandum titled “Maintenance and Distribution of Transgenic Mice Susceptible to Human Viruses” (1993). All animal housing conditions and experiments were approved by Wyeth-Lederle Vaccine and Pediatric’s Animal Care and Use Committee.

Intraspinal inoculation of mice

Four- to six-week-old TGM-PRG-1 and TGM-PRG-3 mice were inoculated IS with the type III poliovirus vaccines 3B and WHO/III. The mice were anesthetized with 10 mg/mL ketamine and 0.4 mg/mL xylazine administered intraperitoneally (ip) at a rate of 300–500 μL/g of body weight. When the anesthetized mice lost pedal reflexes, the lumbar regions in the spinal cords were exposed by cutting the skin aseptically, and 5 μL of virus inoculum was delivered to the gray matter of the spinal cord between the 13th thoracic and 1st lumbar vertebrae. A customized 33-gauge needle (15°) and Exmire microsyringe obtained from the Ito Corporation (Japan) were used to administer virus. After inoculation, the incision was glued with Vetbond (3M Company). The mice were observed for 14 days for development of clinical signs of poliomyelitis (Dragunsky et al., 1996).

Statistical analysis

Paralysis and mortality 50% endpoints were calculated by the Spearman–Karber method. To compare neurovirulent effects of the vaccine 3B and WHO/III reference virus, clinical scores and failure times were tested by contingency table and proportional hazard analyses, respectively. Calculations were performed with the JMP statistical program for the Macintosh computer to estimate likelihood ratio χ² values, factor effects, and probabilities (Lehman and Sall, 1994). For generating contingency tables, infectivity dose, sex, and virus type were used as classifying variables to specify categories in the analysis (Goodman, 1978). In each experiment, the hypothesis that the clinical score is independent of vaccine was tested. Failure time was analyzed as right-censored data in proportional hazard tests (Kalbfleisch and Prentice, 1980). Logistic regression was used to compare dose–responses of the mouse lines.

Detection of pvr in membrane fractions of transgenic CNS tissues

TGM-PRG-1, TGM-PRG-3, and nontransgenic mice were first anesthetized and perfused with PBS (GIBCO-BRL) to remove the blood. The brain, brain stem, and spinal cords were homogenized separately in homogenizing buffer [0.025 M sucrose, 1 mM EDTA, pH 8, 20 mM Tris–HCl, pH 7.2, and protease inhibitor cocktail tablets (Boehringer Mannheim)] with a polytron homogenizer (Brinkmann Instruments) to prepare 10% emulsions (w/v). The homogenates were centrifuged at 400g for 5 min (4°C), then the supernatants were centrifuged at 14,000g, for 30 min at 4°C. 250 μL of homogenizing buffer, plus 1% NP40, were added to the pellets of the second spin (membrane fraction), incubated on ice for 1 h, then centrifuged at 14,000g, for 30 min (4°C). Protein concentrations of the solubilized membrane fractions (i.e., supernatants) were determined.
using a micro-BCA assay (Pierce). Fifteen micrograms of total protein per sample were heated at 85°C in sample buffer containing 25 mM DTT for 5 min then loaded onto 8% acrylamide gels (Novex). Following electrophoresis, the proteins were transferred to PVDF filters (Novex); the filters were blocked overnight in the presence of 1% blocking reagent (Boehringer Mannheim) plus 5% sheep serum (Sigma). Filters with the transferred proteins from the three mouse lines were incubated for 3–4 h with monoclonal antibodies to pvr (1:10 dilution hybridoma cell culture supernatant of 5H5; Koike et al., 1994a) plus actin (1:50; Sigma A4700) in blocking reagent with gentle shaking at room temperature. After being washed twice with TBS, containing 0.1% Tween 20, followed by 0.5% blocking reagent, for 10 min each at room temperature, the filters were incubated with 714 μM/mL sheep anti-mouse/anti-rabbit IgG horse-radish peroxidase (Boehringer Mannheim) for 30 min at room temperature. The filters were washed four times with TBS, containing 0.1% Tween 20, for 15 min each at room temperature. The abundances of pvr and actin were visualized using a Boehringer Mannheim Chemiluminescence Western blotting kit (Cat. No. 1520709) and a 30-min exposure to Bio-Max ML film (Kodak). The actin and pvr protein bands were quantitated using a GS-700 densitometer (Bio-Rad).

ACKNOWLEDGMENTS

We acknowledge Dr. A. Nomoto for the generous gift of 5H5, a monoclonal antibody to pvr, Ms. D. Butler for performing the MAPREC assay to determine the percentage of 472-C in our poliovirus samples, and Dr. T. Zamb for a critical review of the manuscript.

REFERENCES


