Peroral vaccination with a temperature-sensitive mutant of parainfluenza virus type 1 protects mice against respiratory challenge infection

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Peroral vaccination for preventing respiratory infectious diseases was investigated in a murine model using a temperature-sensitive (ts) mutant of parainfluenza virus type 1. The ts mutant virus administered perorally in drinking water neither multiplied nor caused lesions in the respiratory tract or the central nervous system. However, ts virus antigen-positive cells appeared in oropharyngeal lymphoid tissues. This type of antigenic stimulation was capable of inducing both humoral and cellular immune responses, together with an augmentation of interferon production and natural killer cell activity, making it possible to protect the mice against challenge infection with a virulent wild-type virus. These results suggest that the oral cavity, a constituent member of the common mucosal immune system, is a candidate organ applicable as a vaccine route against virus respiratory diseases.

Introduction

Human parainfluenza viruses (PIV) are common respiratory pathogens and often cause severe respiratory diseases, including pneumonia and croup in young children (Heilman, 1990). For the prevention of virus respiratory infection, many attempts have been made to develop a live-attenuated intranasal vaccine utilizing temperature-sensitive (ts) and cold-adapted viruses (Chanock & Murphy, 1980; Crowe, 1995). It is expected that a ts mutant virus acquires genetically attenuated characteristics with growth restriction in the lower respiratory tract where the temperature is much higher than the shut-off temperature for virus growth and, furthermore, that the ts virus will still retain the capability of eliciting virus-specific immune responses.

In previous animal model experiments of intranasal vaccination (Kimura et al., 1979a; Iwata et al., 1990; Tagaya et al., 1995), the ts mutant of PIV type 1 (PIV-1), which had been isolated from a persistently virus-infected cell culture (Kimura et al., 1975), induced both humoral and cellular immunity and successfully protected animals from wild-type (wt) PIV challenge. This ts mutant virus cannot multiply at a temperature higher than 36 °C because of defective virus matrix protein synthesis (Kimura et al., 1979b). However, soon after, it was found that PIV-1, and even its highly attenuated ts derivative, directly access the central nervous system (CNS) by infecting the olfactory neurons (Mori et al., 1995, 1996). The nasal cavity is the only site where central neurons are exposed to the external environment. In light of this fact, more careful consideration of the neurotropism of the vaccine virus should be given in the development of a safer live paramyxovirus vaccine for intranasal use. To avoid possible CNS complications due to the intranasally inoculated vaccine virus, alternative routes for vaccination, such as the alimentary tract and genitourinary tract, can be adopted. This strategy is based on the concept of a common mucosal immune system (Mestecky et al., 1978; McDermott & Beinenstock, 1979; Weisz-Carrington et al., 1979, 1987; Pierce & Cray, 1981; Waldman & Bergmann, 1989), in which immune competent cells that are initially sensitized at one site of a mucous membrane can migrate to other, more distant lymphoid tissues and act as effector cells.

Herein, we describe the immune responses induced by perorally administrating the ts mutant of PIV-1 through drinking water and its protective efficacy against respiratory challenge infection with the homologous wt virus. The positive control of intranasal vaccination has been used throughout as a reference point.
Methods

■ Viruses. The Nagoya strain of PIV-1 and its ts derivative were propagated routinely by allantoic inoculation of 10-day-old embryonated hens’ eggs with 0.2 ml of 10^-1 diluted seed virus. Infected eggs were incubated at 32 °C for the ts mutant and at 38 °C for the wt virus (Kimura et al., 1975). Virus infectivity was assayed by plaque titration on LLCMK2 cell monolayers, as described previously (Kimura et al., 1976). UV-inactivated virus was prepared as described previously (Iwata et al., 1990). After irradiation, infectivity was reduced to < 10^-6 of the original, while haemagglutinin and neuraminidase activities were well preserved.

■ Mice. Specific-pathogen-free, 5-week-old male C3H/Hej mice (Clea) were purchased and acclimatized for 1 week before use. Mice had fresh water and autoclaved food and were kept at 23 °C under biolean conditions throughout all experiments. To avoid laboratory contamination, all virus-infected mice were housed in negatively pressurized isolators equipped with a ventilation system through a high-efficiency particulate air filter (AH model; Nihon-Ika). This work was approved by the Institutional Animal Care and Use Committee of Fuku University, Japan.

■ Experimental infection. For peroral vaccination, a natural feeding method was employed. Overnight, a mouse drank about 6 ml of virus suspension in drinking water containing 5 × 10^5 p.f.u. of the ts mutant. The half-life of the ts virus in the water at room temperature was 12.5 h. For intranasal inoculation, mice were mildly anaesthetized with diethyl ether and inoculated into the right nostril with 20 µl of the ts mutant at a dose of 2.5 × 10^6 p.f.u. per mouse. For intragastric immunization, the ts mutant was given directly into the stomach by intubation through a gastric tube. After 3 weeks, mice were challenged intranasally with 0.3 × 10^6 p.f.u. of wt virus. Samples were then collected at regular intervals.

■ Immunohistochemical procedure. Mice were anaesthetized and perfused with 4% paraformaldehyde. The periglandular and cervical lymph nodes were removed and embedded in Tissue-Tek OCT compound (Sakura Finetek). After being snap-frozen with dry ice–methanol, the periglandular and cervical lymph nodes were incubated for 2 h at 37 °C under bioclean conditions throughout all experiments. To avoid laboratory contamination, 10% foetal calf serum and 5 × 10^-3 M 2-mercaptoethanol. Lyophilized were restituted in vitro by co-cultivating for 5 days with mitomycin C (Biomol Research Laboratories) treated syngeneic spleen cells that had been infected with wt virus 1 h before. L929 cells infected with wt virus at an input m.o.i. of 1 p.f.u. were used as target cells. The L929 cell line was originally derived from a C3H mouse and both L929 and spleen tested cell the same H-2 haplotype. Lymphocytes and target cells were mixed and incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. Specific lysis of target cells was determined by the lactate dehydrogenase-release assay (Decker & Lohmann-Matthes, 1988) using a cytopotoxicity detection kit (Roche). Data were expressed as the percentage of specific release using the following formula:

\[ \text{cytotoxicity} (%) = \frac{[(\text{target effector} - \text{target spontaneous}) - \text{target spontaneous}]}{\text{target maximum} - \text{target spontaneous}} \times 100 \]

■ Assay of natural killer (NK) cell activity. Yac-1 target cells were incubated with spleen cells at 37 °C for 4 h. Specific lysis of target cells was determined by the lactate dehydrogenase-release assay.

■ Assay of interferon (IFN) activity. The antiviral activity of IFN was measured by a microassay method using mouse L929 cells and vesicular stomatitis virus as the challenge virus (Iwata et al., 1990). The highest dilution of the test serum causing complete protection against the cytopathic effects of the challenge virus was defined as containing 1 unit of IFN. In our laboratory, 1 IFN unit is equivalent to 2.7 reference research units of a reference preparation of mouse IFN (NIH, catalogue no. G002-904-511).

■ Statistical analysis. Data represent the mean ± SD, which are expressed as geometric means. The two-tailed Mann–Whitney U-test was done to determine whether there was a significant difference (P < 0.05) between the experimental and control groups.

Results

Virus antigens in the oropharyngeal lymph nodes

Mice were inoculated perorally through drinking water with 5 × 10^5 p.f.u. per mouse of the ts mutant of PIV-1. At 3 days after administration, immunohistochemistry assays disclosed the presence of virus antigen-positive cells in the periglandular and cervical lymph nodes (Fig. 1). These antigen-containing cells distributed mostly in the rim of the lymphoid follicles. It is of note that no infectious progeny virus could be isolated by plaque titration in the nasal turbinates, tracheas or lungs during the course of infection. Sensitive nested PCR detected no virus genomes in either the lungs or the nasal
Peroral parainfluenza virus vaccine

Fig. 1. Immunohistochemical detection of virus antigens in the periglandular lymph nodes of mice vaccinated perorally with the ts mutant of PIV-1. (A) Virus antigen-positive cells (arrows) at 3 days after peroral vaccination. (B) Mock-infected control mouse. Bar, 20 µm.

Fig. 2. Agarose gel electrophoresis of nested PCR products of the nucleoprotein gene of PIV-1. The arrow indicates a 340 bp product. Lanes 1, marker DNA; 2, wt virus (positive control); 3, the lungs of mice intranasally vaccinated with the ts mutant; 4 and 5, the lungs and the nasal turbinates, including the olfactory bulbs, of mice perorally vaccinated, respectively.

Specific antibody response

The kinetics of specific antibody production in mice orally immunized with the ts mutant was investigated by ELISA. The titre of serum IgG antibody became detectable at 7 days and reached a plateau at 2 weeks after vaccination. Antibody titres were maintained at a high level for the long period of more than 4 weeks of testing (Fig. 3). At 3 weeks after vaccination, the major class of antibody with significant neutralizing activity was IgG (Table 1). The serum neutralizing antibody titres were $10^6 \pm 1 \times 10^1$ and $40 \times 1 \pm 2$ for peroral and intranasal vaccination, respectively. Intranasal vaccination could induce more elevated IgG and IgA antibody responses, especially in the bronchoalveolar lavage fluid (Table 1). When mice that had been primed 3 weeks before were challenged intranasally with wt virus, increased antibody response could be detected in both groups of mice with peroral and intranasal routes of priming (Table 1). This augmentation is quite certainly due to a booster effect from wt virus challenge, as no measurable antibody production could be detected yet in unprimed mice at 5 days after challenge. These results indicate that peroral vaccination is an effective procedure for eliciting humoral immune responses.

Virus-specific CTL response

Specific CTL activity induced in mice 10 days after peroral vaccination with the ts mutant was almost equivalent to that of intranasal vaccination. The specific cytotoxicity values at an effector:target cell ratio of 50:1 were $27.7 \pm 7.9$ and $35.0 \pm 6.7$% for peroral and intranasal vaccination, respectively. When mice primed with the ts mutant 3 weeks before were challenged with wt virus, the specific CTL cytotoxicity appeared more quickly, with a predominantly enhanced activity in both routes of immunization (Fig. 4). The memory function of the CTL response induced by peroral vaccination lasted for at least the 3 months tested, although it diminished slightly faster than that of intranasal vaccine priming (Fig. 5).

IFN production and NK cell activity

At an early stage of infection, before the appearance of specific immune responses, IFN and NK cells are the major factors that contribute to a rapid termination of virus infection. The antiviral activity of IFN was detected in mice orally
Table 1. Antibody responses in mice immunized with the ts mutant of PIV-1

Samples were collected at 3 weeks after vaccination and at 5 days after wt virus challenge of vaccine-primed mice. Data are the mean ± SD of results for each group of six mice tested.

<table>
<thead>
<tr>
<th>Route of vaccination</th>
<th>Challenge infection</th>
<th>Serum</th>
<th>Bronchoalveolar lavage fluid</th>
<th>Nasal wash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
<td>IgM</td>
</tr>
<tr>
<td>Peroral</td>
<td>None</td>
<td>2.29 ± 0.39</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>3.45 ± 0.22*</td>
<td>&lt; 1.30</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>Intrasoral</td>
<td>None</td>
<td>3.37 ± 1.36</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>4.30 ± 0.13</td>
<td>1.47 ± 0.17</td>
<td>1.87 ± 0.38</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
<td>&lt; 1.30</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.01 by Mann–Whitney U-test) compared with corresponding intranasally vaccinated mice.

Table 2. IFN production in mice inoculated with the ts mutant of PIV-1

Serum samples were collected at 2 days after inoculation of mice with the ts mutant or at 2 days after wt virus challenge of primed mice that had been vaccinated 1 day before. Data are the mean ± SD of results for each group of four mice tested.

<table>
<thead>
<tr>
<th>Route of vaccination</th>
<th>Challenge infection</th>
<th>Serum IFN titre (log$_{2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroral</td>
<td>None</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>4.8 ± 0.8*</td>
</tr>
<tr>
<td>Intrasoral</td>
<td>None</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>5.2 ± 1.0*</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td></td>
<td>wt virus</td>
<td>2.1 ± 0.0</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.05 by Mann–Whitney U-test) compared with corresponding unvaccinated control mice.

vaccinated with the ts mutant 2 days before. In both intranasal and peroral routes of vaccination, primed mice produced more satisfactory amounts of IFN in response to wt virus challenge when compared with unprimed mice (Table 2).

NK cell activity could also be detected in mouse spleen cells at about 3–5 days after vaccination. The intranasal route induced more powerful cytotoxicity of NK cells.

Protective capacity of peroral vaccination against wt virus challenge

In order to evaluate the protective capacity of vaccination, mice were administered perorally with the ts mutant and 3
weeks later challenged intranasally with wt virus. The growth of the challenge virus in the lungs was completely inhibited (Table 3). The prophylactic efficacy was comparable to that of intranasal vaccination. When the ts mutant was introduced at a dose of $5 \times 10^3$ p.f.u./0.1 ml into the stomach directly through a gastric tube, no virus growth in the upper and lower digestive tract tissues could be detected by PCR and no successful protection against wt virus challenge could be achieved. The protective capacity of the ts mutant through the peroral route was removed when the virus was inactivated by a large dose of UV irradiation.

**Table 3. Protective efficacy of the vaccination with the ts mutant of PIV-1**

Vaccinated mice were challenged intranasally with wt virus and 5 days later assayed for infectivity in the lungs at 38 °C. Data are the mean ± SD of results for each group of six mice tested.

<table>
<thead>
<tr>
<th>Route of vaccination</th>
<th>Growth of wt virus (log p.f.u. per lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroral</td>
<td>$&lt; 0.5^*$</td>
</tr>
<tr>
<td>Peroral (UV-inactivated)</td>
<td>$3.8 \pm 0.7$</td>
</tr>
<tr>
<td>Intranasal</td>
<td>$&lt; 0.5^*$</td>
</tr>
<tr>
<td>Intragastric</td>
<td>$4.1 \pm 0.2$</td>
</tr>
<tr>
<td>None</td>
<td>$4.3 \pm 0.5$</td>
</tr>
</tbody>
</table>

* Significant difference ($P < 0.01$ by Mann–Whitney U-test) compared with corresponding unvaccinated control mice.

For induction of protective immune responses, replication of the vaccine virus and/or synthesis of virus structural proteins are essential, since the UV-inactivated virus proved to be ineffective (Table 3). The invalidity of a direct intragastric route of vaccination might be due to destruction of the vaccine virus by the acid in gastric juice. From the results of the preliminary experiment, an inoculum dose of more than $5 \times 10^5$ p.f.u. per mouse in drinking water was needed for protection. The replication of orally administered vaccine virus possibly occurs in mucous membrane cells of the alimentary tract above the stomach, namely the oral cavity, the pharynx and the oesophagus. In the present peroral vaccination, the intestinal lymphoid tissues (Nedrud *et al.*, 1987) might not be involved. Actually, virus antigen-positive cells are detected in the oropharyngeal lymph nodes that are draining from the site of antigen exposure. Even at non-permissive temperatures, these ts mutant-infected cells produce virus proteins, such as haemagglutinin and neuraminidase protein and fusion protein (Kimura *et al.*, 1979b); the former binds to the cellular receptors and the latter functions during the fusion process between the virus envelope and host cell membrane. Both envelope spike proteins act at the first indispensable step of virus penetration into the cell. Therefore, the immune responses to them play a decisive role in a host defence system.

Paramyxovirus is transmitted naturally by droplets and spreads throughout the respiratory tract. It is reasonable to consider that respiratory immunity can be established by intranasal vaccination. However, even the attenuated vaccine strain of PIV-1 accesses the CNS by infecting the olfactory neurons as well as in the respiratory tract grants the peroral route of vaccination an advantage in warding off an unfavourable involvement with CNS complications. Thus, peroral delivery of PIV vaccine attains definite points of superiority over the intranasal route as follows: (i) the induction of sufficient prophylactic efficacy by sensitizing a mass of lymphoid tissues associated with the orogastrointestinal tract; (ii) increased safety in terms of CNS involvement; (iii) no infliction of pain on the recipient; and (iv) cheaper and easier handling without any equipment like syringes and nebulizers.

It is tempting to speculate that a strategy of peroral vaccination with a live attenuated virus could be applied to respiratory infectious diseases, such as measles, mumps, rubella and influenza.

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**References**


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