Expression of the HN, F, NP and M proteins of Sendai virus by recombinant vaccinia viruses and their contribution to protective immunity against Sendai virus infections in mice

Takemasa Sakaguchi, Shin-ichi Takao, Katsuhiro Kiyotani, Yutaka Fujii, Takayasu Nakayama and Tetsuya Yoshida*

Department of Bacteriology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

Recombinant vaccinia viruses (RVVs) expressing each of the haemagglutinin–neuraminidase (HN), fusion (F), nucleocapsid (NP) and matrix (M) proteins of Sendai virus were constructed to investigate their capacities to induce protective immunity against Sendai virus infections. The proteins expressed in cultured cells appeared to be authentic with respect to their antigenicity, electrophoretic mobility, surface expression of the HN and F proteins and, in the case of the HN protein, biological activities. Mice inoculated intranasally with these RVVs developed serum antibodies to the respective Sendai virus proteins, suggesting their in vivo expression. In mice immunized with RVV carrying either the HN or the F gene, growth of the challenging Sendai virus was almost completely suppressed in the lung, indicating their capacities to induce effective protective immunity against Sendai virus infections. In contrast, in mice immunized with RVV carrying the NP or M gene, the challenging virus propagated as well as in the control mice, but the virus titres were significantly lower at the late stage of infection than those in the control mice, suggesting that they can also induce protective immunity especially at the late stage of the challenge infection.

Sendai virus, a prototype of the paramyxovirus family, consists of a helical ribonucleoprotein, the nucleocapsid, which is surrounded by an envelope. The nucleocapsid consists of the ssRNA genome and the major subunit protein (NP). The envelope contains two types of spike glycoproteins on its surface; one is HN, which has haemagglutinin and neuraminidase activities, and the other is F, which induces cell fusion and haemolysis. Both glycoproteins play essential roles in virus attachment to the cell surface and penetration of the viral genome into target cells, respectively. The inner side of the envelope is coated with the matrix (M) protein, which plays a crucial role during virus assembly (Kingsbury, 1990). Sendai virus is a causative agent of typical local infections of the respiratory tracts of rodents, and often causes outbreaks of fatal pneumonia in laboratory mice (Ishida & Homma, 1978). There are many reports about protection of mice from Sendai virus infections by the use of killed and live vaccines. These include inactivated virus (Fukumi & Takeuchi, 1975), a synthetic oligopeptide corresponding to the partial amino acid sequence of NP protein (Kast et al., 1991), F protein-cleavage mutants (Tashiro & Homma, 1985; Itoh et al., 1990) and a temperature-sensitive mutant possessing homologous interfering capacity (Kimura et al., 1979; Kiyotani et al., 1990).

Immunization with recombinant vaccinia viruses (RVVs) has the advantage of eliciting an effective cellular as well as humoral immune response to the expressed antigen (Mackett et al., 1984), and its usefulness was examined in viral respiratory infections such as those caused by influenza virus (Panicali et al., 1983; Smith et al., 1983), human parainfluenza virus type 3 (Spriggs et al., 1987) and respiratory syncytial virus (RSV) (Elango et al., 1986; Olmsted et al., 1986; Stott et al., 1986), but not in Sendai virus infections. In the present study we constructed RVVs expressing the HN, F, NP or M protein of Sendai virus to evaluate their capacities to induce protective immunity against Sendai virus infections in mice.

Vaccinia virus strain WR and RVVs were propagated in HEp-2 cells. The Z strain and the Hamamatsu strain of Sendai virus were grown in embryonated eggs. The Hamamatsu strain is a wild-type strain freshly isolated from a laboratory infection and propagates well in the mouse lung (Kiyotani et al., 1990), and was used as a challenge virus. The infectivity of vaccinia virus was assayed by the plaque method using LLCMK₂ cells and
Short communication

Fig. 1. Synthesis of Sendai virus proteins by the RVVs. Monolayers of LLCMK2 cells in 3.5 cm dishes were infected with Sendai virus Z strain (lane 1), vaccinia virus WR strain (lane 2), Vac-HN (lane 3), Vac-F (lane 4), Vac-NP (lane 5), Vac-M (lane 6) or mock-infected (lane 7) at an input multiplicity of approximately 5 p.f.u. per cell. After an adsorption period of 1 h, the cells were incubated in MEM for 5 h, and the medium was replaced with MEM deficient in methionine. After a 15 rain starvation, the cells were labelled for 1 h with 10 laCi/ml of L-[³⁵S]methionine (> 1000Ci/mmol, American Radiolabeled Chemicals). The cells were then solubilized with lysis buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.15 M-NaCl, 50 mM-Tris-HCl pH 8.0, 1 mM-PMSF), and the cell lysates were clarified by centrifugation at 15000 r.p.m, for 20 rain. Sendai virus proteins in the supernatants were immunoprecipitated using anti-Sendai virus antiserum and fixed Staphylococcus aureus, followed by 10 % SDS-PAGE and fluorography.

that of Sendai virus was measured by the immunofluorescent cell-counting method using LLCMK2 cells (Kashiwazaki et al., 1965). These infectivities were expressed as p.f.u. and cell-infecting units (c.i.u.), respectively. HEp-2 and LLCMK2 cells were propagated in Eagle’s MEM supplemented with 10% and 5% newborn calf serum, respectively.

Sendai virus Z strain cDNA, encompassing the open reading frames of the NP, M, HN or F proteins, was constructed from plasmids made from genomic RNA (Hidaka et al., 1984; Shioda et al., 1983, 1986). The cDNA was inserted into the multiple cloning site of the vaccinia virus expression plasmid pNZ68K2, which contains the promoter sequence of the 7.5K protein upstream of an inserted multiple cloning site and the thymidine kinase gene. The construction of recombinant viruses was performed as described (Gotoh et al., 1989). The RVVs carrying the NP, M, HN and F genes of Sendai virus were designated Vac-NP, Vac-M, Vac-HN and Vac-F, respectively.

To examine expression of the proteins, monolayers of LLCMK2 cells were infected with each RVV, and labelled with [³⁵S]methionine. The Sendai virus proteins synthesized were precipitated with anti-Sendai virus polyclonal antiserum, and analysed by SDS–PAGE and autoradiography. As shown in Fig. 1, discrete protein bands were found in the gel at the same positions as their authentic counterparts in the Z strain. The mobility of each protein in the gel and their reactivity with the antiserum suggest the expression of authentic Sendai virus proteins by the RVVs. The authenticity of the epitopes in the expressed HN and F proteins was further defined by indirect immunofluorescent staining of Vac-HN- and Vac-F-infected cells using monoclonal antibodies recognizing distinct antigenic sites of the glycoproteins, HN-43, HN-228, HN-312, HN-892, F-49, F-128 and F-881 (Tozawa et al., 1986). All the antibodies recognized the respective proteins expressed in infected LLCMK2 cells (data not shown).

Haemadsorption on Vac-HN-infected cells was further examined. LLCMK2 cells infected with the WR strain of vaccinia virus adsorbed chicken erythrocytes on their surface by the viral haemagglutinin (Fig. 2a) as described (Stone & Burnet, 1946), but did not adsorb guinea-pig erythrocytes (Fig. 2b). Hence haemadsorption by the HN proteins expressed by Vac-HN could be distinguished from that caused by the vaccinia virus haemagglutinin by using guinea-pig erythrocytes. As shown in Fig. 2(c), cells infected with Vac-HN adsorbed guinea-pig erythrocytes, indicating that the expressed HN proteins have haemagglutinating activity and are transported to the cell surface.

The neuraminidase activity of Vac-HN-infected cell lysates was also examined. Infected cells in a 3.5 cm dish were washed twice with PBS, frozen and thawed in 500 μl of 0.9 % NaCl and sonicated for 30 s. A 50 μl sample of the cell lysate was mixed with an equal volume of 24 mg/ml fetuin (Sigma), and incubated at 37 °C for 18 h. The free sialic acid in the sample was coloured by the method of Warren (1959). The optical density at 549 nm of the Vac-HN-infected cell lysates was 0.535 (mean of duplicate measurements), whereas values for WR strain-infected and uninfected cell lysates were 0.007 and 0.004, respectively, indicating that the expressed HN proteins have neuraminidase activity.

When monolayers of LLCMK2 cells infected with Vac-F were labelled for 20 min with [³⁵S]methionine and chased for 90 min in medium containing trypsin, significant amounts of the F protein precursors (F₀) synthesized were converted to F₁ (data not shown). This
indicates that the expressed F proteins are susceptible to proteolytic cleavage by trypsin in the medium and that significant amounts of the proteins are transported to the cell surface. But multinuclear cell formation, an indicator of the fusion activity of the F protein, was observed neither in LLCMK₂ nor in BHK-21 cells when infected with Vac-F in the presence of trypsin. This was the case even when LLCMK₂ or BHK-21 cells were co-infected with Vac-F and Vac-HN. It is not clear why the F protein expressed by Vac-F showed no cell fusion activity. One possible explanation for this lack of activity is that severe cytopathic effects of vaccinia viruses may disturb the expression of the activity, as suggested for the F proteins of RSV and human parainfluenza virus type 3 expressed by RVVs (Spriggs et al., 1987; Wertz et al., 1987).

Expression of Sendai virus proteins in mice by the RVVs was examined by induction of antibodies to the respective proteins. An RVV carrying the HN gene of Newcastle disease virus, Vac-HN(NDV) (Nishino et al., 1991), was used as a negative control, because the WR strain was lethal under the present experimental conditions probably due to neurovirulence through its thymidine kinase (Buller et al., 1985). The serum antibody produced was examined by immunoprecipitation of Sendai virus-infected cell lysates and SDS-PAGE. Each serum precipitated the respective Sendai virus protein (data not shown), suggesting that the Sendai virus proteins are correctly expressed in mice by the RVVs.

The serum antibodies were also assayed by ELISA, haemagglutinin inhibition (HI) and virus neutralization. As shown in Table 1, the RVVs carrying Sendai virus genes induced significant amounts of ELISA antibodies to Sendai virus, but the antibody titre of the Vac-M-infected mice was lower than those of the other mice. Virus neutralization activity was detected in sera from mice inoculated with Vac-HN or with Vac-F but not in those from mice inoculated with Vac-NP or with Vac-M. This was the case even in the presence of complement, although a significant enhancement of neutralization was

![Fig. 2. Haemadsorption on cells infected with Vac-HN. Monolayers of LLCMK₂ cells were infected with vaccinia virus WR strain (a, b) or Vac-HN (c) at an input multiplicity of 2 p.f.u. per cell, and incubated for 20 h at 37 °C. The infected cells were washed twice with PBS, and then overlaid with 2% chicken (a) or guinea-pig (b, c) erythrocytes in PBS. After incubation for 15 min at 4 °C, the cells were washed five times with PBS, and observed under a microscope.](image-url)
Table 2. Protection of mice from Sendai virus infection by immunization with RVVs

<table>
<thead>
<tr>
<th>RVV used for immunization*</th>
<th>Sendai virus titre in the lung after challenge infection (log10 c.i.u./ml)</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vac-NP</td>
<td>ND†</td>
<td>7.6</td>
<td>6.3</td>
<td>&lt; 3.1</td>
<td></td>
</tr>
<tr>
<td>Vac-M</td>
<td>ND†</td>
<td>7.6</td>
<td>6.4</td>
<td>&lt; 3.1</td>
<td></td>
</tr>
<tr>
<td>Vac-HN</td>
<td>ND†</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
</tr>
<tr>
<td>Vac-F</td>
<td>ND†</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
<td>&lt; 3.1</td>
</tr>
<tr>
<td>Vac-HN(NDV)</td>
<td>&lt; 3.1</td>
<td>7.4</td>
<td>7.5</td>
<td>&lt; 3.1</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were inoculated intranasally with each RVV as described for Table 1. Three weeks after inoculation, mice were intranasally challenged with 10⁶ c.i.u. of the Hamamatsu strain in 25 µl PBS, and two mice were sacrificed on various days thereafter. Lungs were homogenized in 1 ml MEM, centrifuged at low speed and infectivity in the supernatant was measured (Kiyotani et al., 1990). Each value is a geometric mean.
† ND, Not determined.

observed in serum from Vac-F-infected mice. The serum from Vac-HN-inoculated mice also showed HI activity.

At 3 weeks after immunization with each of the RVVs, the mice were intranasally challenged with 10⁶ c.i.u. of the Hamamatsu strain of Sendai virus to evaluate their protective capacities. Two mice of each group were sacrificed 0, 5, 7 and 10 days after the challenge infection, and infectivity of Sendai virus in the lungs was measured by the immunofluorescent cell-counting method (Table 2). As shown in the control mice immunized with Vac-HN(NDV), the challenge virus grew well in the lung, reaching a maximum on the 5th day and disappearing by the 10th day. However, the mice immunized with Vac-HN or Vac-F did not permit replication of the challenge virus in the lung. In contrast, in mice immunized with Vac-NP or Vac-M, the challenge virus propagated until 5 days after infection as well as in mice immunized with Vac-HN(NDV), but the virus titres on the 7th day after infection seemed to be lower than that in the control mice. Neither lung consolidation nor body weight loss was observed throughout the challenge infection, either in mice immunized with RVVs carrying Sendai virus genes or in control mice. The suppression of the challenge virus growth in the lung is not considered to be due to in vitro neutralization of virus infectivity by contaminated serum during material preparation, because almost no reduction of the infectivity was observed when lung homogenates of mice immunized with Vac-HN or Vac-F were mixed in vitro with known amounts of infectious Sendai virus (data not shown). Further, in the case of immunization with Vac-NP or Vac-M, the antibodies to NP and M showed no virus-neutralizing activity even in the presence of complement (Table 1).

To confirm the suppression of challenge virus growth observed in Vac-NP- or Vac-M-inoculated mice, a similar experiment was carried out using five mice per group. As shown in Table 3, the mean titres of challenge virus infectivity in the lungs of Vac-NP- or Vac-M-inoculated mice were lower on the 7th and 8th days than those of the control mice, and the difference in infectivities on the 8th day was statistically significant between the Vac-NP- or Vac-M-inoculated mice and the control mice.

The present results indicate that Vac-HN and Vac-F can induce effective protective immunity against Sendai virus infections, and suggest that Vac-NP and Vac-M can also induce protective immunity especially at the late stage of the challenge infection. These results are essentially compatible with those of previous studies on protection by RVVs against respiratory infections by other ortho- and paramyxoviruses. In these studies, RVVs expressing the surface glycoproteins of influenza virus (Panicali et al., 1983; Smith et al., 1983), human parainfluenza virus type 3 (Springgs et al., 1987) and RSV (Elango et al., 1986; Olmsted et al., 1986; Stott et al., 1986; Johnson et al., 1987; Wertz et al., 1987) induced strong protective immunity against the respective viral infections. In contrast, RVVs expressing internal proteins, NP in the case of influenza virus (Andrew et al., 1987; Endo et al., 1991) and M2 and N in the case of RSV (King et al., 1987; Connors et al., 1991), caused weak protective immunity, as suggested by reduction of the virus in the lung. It has been demonstrated that mice immunized with RVVs expressing the G, F or N proteins of RSV show significantly greater histological lung lesions than unimmunized mice after challenge infection with RSV (Stott et al., 1987). We observed, however, neither lung consolidation nor body weight loss during challenge infection by Sendai virus in mice immunized with RVVs carrying Sendai virus genes, although lesions in the lung were not examined microscopically in the present study.

Table 3. Protection of mice from Sendai virus infection by immunization with Vac-NP or Vac-M

<table>
<thead>
<tr>
<th>RVV used for immunization*</th>
<th>Sendai virus titre in the lung after challenge infection (log10 c.i.u./ml)</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vac-NP</td>
<td>7.1 ± 0.3</td>
<td>5.2 ± 1.3</td>
<td>3.0 ± 0.3†</td>
<td></td>
</tr>
<tr>
<td>Vac-M</td>
<td>7.0 ± 0.3</td>
<td>5.4 ± 1.0</td>
<td>&lt; 2.8 ± 0‡</td>
<td></td>
</tr>
<tr>
<td>Vac-HN(NDV)</td>
<td>6.9 ± 0.3</td>
<td>6.1 ± 0.6</td>
<td>4.3 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were immunized with each RVV, and then challenged with the Hamamatsu strain as described for Table 2. Five mice were sacrificed on the day indicated, and Sendai virus infectivity in the lung was assayed. The infectivity is expressed as the mean ± standard deviation.
† Significant protection at P = 0.0015 in one-way analysis of variance and Scheffe's multiple comparison.
‡ Significant protection at P = 0.004.
The mechanism of the protective immunity to Sendai virus induced by the RVVs in the present study is unknown. The strong suppression of challenge virus growth in the lung of animals immunized with Vac-HN or Vac-F might be due to antibodies produced in the respiratory tract in addition to exuded serum antibodies, since antibodies in both sera against HN and F showed strong virus neutralizing activity in vitro. On the other hand, the reduction of challenge virus growth at the late stage in Vac-NP- or Vac-M-immunized mice is probably due to cytotoxic T lymphocytes against NP and M, because the antibodies to the internal proteins showed no neutralizing activity even in the presence of complement. The mechanisms responsible for the protective immunity observed in the present study should be further clarified.

We thank Dr H. Shibuta, Institute of Medical Science, The University of Tokyo, for donating the cDNA of Sendai virus Z strain, Nippon Zoon for the vaccinia virus expression plasmid pNZ68K2, Dr H. Tozawa, Kitasato University, for monoclonal antibodies against the Sendai virus HN and F proteins, and Dr Y. Nagai, Nagoya University School of Medicine, for the RVV carrying the HN gene of NDV. We also thank A. Soo, Hiroshima University School of Medicine, for his valuable suggestions on statistical analysis. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a grant from the Tsuchiya Foundation.

References


FUKUMI, H. & TAKEUCHI, Y. (1975). Vaccination against parainfluenza 1 virus (type mumps) infection in order to eradicate this virus in colonies of laboratory animals. Developments in Biological Standardization 28, 477–481.


Short communication


(Received 2 September 1992; Accepted 4 November 1992)