Homotypic and heterotypic protection against influenza virus infection in mice by recombinant vaccinia virus expressing the haemagglutinin or nucleoprotein gene of influenza virus

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Recombinant vaccinia virus expressing the influenza virus haemagglutinin (HA) or nucleoprotein (NP) genes from A/SW/Hong Kong/1/74 (H1N1) under the control of a hybrid promoter containing the P7.5 early promoter element and promoter of the gene encoding the major protein of cowpox virus A type inclusion body was constructed to investigate protective immunity against homologous and heterologous viruses in mice. These recombinant vaccinia viruses produced authentic influenza virus HA and NP in infected cells. The recombinant vaccinia virus–influenza virus HA conferred efficient subtype-specific protection although mice challenged with heterologous influenza viruses underwent initial infection. By contrast, immunization with the recombinant vaccinia–influenza virus NP limited virus multiplication in the lungs against challenge infection with all H1N1 and H3N2 influenza viruses examined, although less efficiently. These results will prompt the re-examination of the possibility of using the recombinant vaccinia virus–influenza virus NP as a cross-protective vaccine

Antibody-mediated immunity to influenza virus is strain- or subtype-specific, whereas cell-mediated immunity is cross-reactive among type A influenza viruses (Askonas et al., 1982; Ada et al., 1983; Ada & Jones, 1986). The major protective antibody is directed against the envelope glycoprotein haemagglutinin (HA). Influenza virus HA-specific antibody neutralizes infectivity of the virus in vitro and protects mice from challenge infection with homologous virus (Askonas et al., 1982). On the other hand, cytotoxic T lymphocytes (CTL) recognize the nucleoprotein (NP) as a cross-reactive antigen and aid virus clearance from the host (Ennis et al., 1978; Yewdell et al., 1985; Taylor & Askonas, 1986; McMichael et al., 1986; Gotch et al., 1987). Previous studies demonstrated that recombinant vaccinia virus expressing influenza virus HA elicits virus-neutralizing antibody and subtype-specific CTL (Panicali et al., 1983; Smith et al., 1983; Bennink et al., 1984; Coupar et al., 1986), and that recombinants expressing NP stimulate cross-reactive CTL (Yewdell et al., 1985). The protective efficacy of HA and NP against homologous virus has been evaluated; vaccinia–HA recombinant virus affords total protection against lethal challenge infection, but vaccinia–NP virus provides poor protective immunity (Andrew et al., 1987; Andrew & Coupar, 1988; Stitz et al., 1990). Since the antigenicity of influenza virus changes with time, protection against heterologous virus is of considerable importance for the development of an influenza vaccine.

In this study, we constructed new recombinant vaccinia virus expressing either the influenza virus HA or NP gene under the control of a hybrid cowpox–vaccinia virus promoter with enhanced promoter activity and have investigated the protective efficacy of these two recombinant vaccinia viruses against challenge infection with homologous and heterologous influenza viruses.

To construct the recombinant vaccinia viruses, full-length cDNA was synthesized from purified viral RNA of A/SW/Hong Kong/1/74 (SW/HK) (H1N1) by using the universal primer 5'AGCAAAAAGCAGG complementary to the 3' terminus of viral RNA. The cDNA of either the HA or the NP gene was ligated with an EcoRI linker and inserted into the EcoRI site of plasmid pSFB5, which contains the flanking HA gene sequence of vaccinia virus for homologous recombination with the
vaccinia virus genome at the HA locus and four unique sites for the insertion of a foreign gene downstream from a hybrid cowpox–vaccinia virus promoter. The resultant plasmids, pSHA1 and pSNP1, were used to generate recombinant viruses vSHA1 (HA-expressing recombinant virus) and vSNP1 (NP-expressing virus), respectively, as described previously (Itamura et al., 1990). The protein expressed from the recombinant vaccinia viruses was analysed using a radioimmunoprecipitation assay with antisera specific for SW/HK virus. A 74K polypeptide was detected in vSHA1-infected cells, and it appeared similar in size to the glycosylated, uncleaved influenza virus HA. In vSNP1-infected cells, a 56K polypeptide was observed, corresponding to the expected size of influenza virus NP. Immunofluorescence studies indicated that the HA expressed from vSHA1 was transported to the cell surface and, in contrast, the NP expressed from vSNP1 was predominantly localized in the nucleus (data not shown). We observed haemadsorption of chicken erythrocytes and trypsin-activated, acid-inducible cell fusion by vSHA1-infected cells, but not by vSNP1-infected cells (data not shown). Thus the recombinant vaccinia viruses were found to express functionally authentic influenza virus HA from vSHA1 and NP from vSNP1.

To examine the protective ability of the recombinant vaccinia viruses against homologous virus, groups of ddY mice were immunized intravenously (i.v.) with 10^7 p.f.u. of either vSHA1, vSNP1 or insert-free vaccinia virus WR(HA^-), and 21 days later were challenged with an aerosol mist of SW/HK virus by using a jet nebulizer (Nihon Clea). In this experiment, no group of mice showed any symptoms of morbidity or pathological changes in the lung. Table 1 shows virus multiplication in the lungs of immunized mice after challenge with SW/HK virus. All mice immunized with vSHA1 were completely protected from infection. By contrast, immunization with vSNP1 did not protect mice from infection but reduced the lung virus titre on day 7 when compared with the immunization effect of WR(HA^-), although its efficiency was lower than that with vSHA1. We also examined serum neutralizing antibody titres against SW/HK virus in vaccinated mice prior to challenge infection (Table 1). The recombinant virus vSHA1 induced high levels of serum neutralizing antibody in mice but vSNP1 and WR(HA^-) did not produce detectable levels.

To analyse the efficacy of heterotypic protection by recombinant vaccinia viruses, we used two heterologous viruses, A/Yamagata/120/86 (Yam) (H1N1) and A/Sichuan/2/87 (Sic) (H3N2) as challenge viruses. Although the heterologous virus Yam belongs to the same serotype as the homologous SW/HK virus, it is antigenically distinct. The ddY mice (11 per group) immunized with vSHA1, vSNP1 or WR(HA^-) were challenged with an aerosol mist of 3 LD_{50} Yam virus 21 days post-immunization. All mice immunized with vSHA1 survived the lethal challenge but showed some signs of morbidity such as lethargy, hunching, weight loss and rapid respiration. Immunization with vSNP1 partially protected mice from the lethal challenge (73%) and caused morbidity in all mice according to the above criteria. Almost all mice (82%) immunized with WR(HA^-) had died by day 12 with this challenge dose. The weight loss in mice immunized with vSHA1 was recovered earlier than that in mice protected by vSNP1 (data not shown). The challenge virus was detected in the lungs of all mice immunized with vSHA1, vSNP1 or WR(HA^-) on days 3 and 5 (Table 1). However,
Table 1. **Protection from challenge infection of influenza viruses in mice** immunized with recombinant vaccinia viruses

<table>
<thead>
<tr>
<th>Challenged with</th>
<th>Vaccinated with</th>
<th>Neutralizing antibody titre</th>
<th>Pulmonary virus titre*&lt;sup&gt;‡&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt; p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 3</td>
<td>day 5</td>
</tr>
<tr>
<td>SW/HK (H1N1)</td>
<td>vSHA1</td>
<td>8192</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td>vSNP1</td>
<td>&lt;32</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>WR(HA&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>&lt;32</td>
<td>5.3</td>
</tr>
<tr>
<td>Yam (H1N1)</td>
<td>vSHA1</td>
<td>32</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>vSNP1</td>
<td>&lt;32</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>WR(HA&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>&lt;32</td>
<td>7.0</td>
</tr>
<tr>
<td>Sic (H3N2)</td>
<td>vSHA1</td>
<td>&lt;32</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>vSNP1</td>
<td>&lt;32</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>WR(HA&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>&lt;32</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* ddY outbred mice (obtained from Japan SLC) were challenged with the indicated virus 21 days after immunization with the recombinant or the control WR(HA<sup>-</sup>).

† Neutralizing antibody titres against challenge viruses were determined in pooled sera from four to six immunized mice prior to challenge using the 50% reduction of plaque formation assay. The neutralizing antibody titre was expressed as the reciprocal of the serum dilution causing 50% reduction of plaque formation.

‡ Homogenates (2 ml/lung) of three pooled lungs per group were titrated on MDCK monolayer cells essentially as described previously (Tobita et al., 1975).

Multiplication of the challenge virus in the lung was reduced on days 5 and 7 in mice immunized with vSHA1 and vSNP1. The rate of reduction of lung virus titre in vSHA1-immunized mice was greater than that in vSNP1-immunized mice. vSHA1-immunized mice had low titres of serum neutralizing antibody against Yam, but those immunized with vSNP1 and WR(HA<sup>-</sup>) had no detectable levels of antibody. Similar protection was observed when mice were challenged with a low lethal dose of A/PR/8/34 (H1N1) virus which is also antigenically distinct from the homologous SW/HK virus (data not shown).

Next, immunized mice were challenged with Sic, which has a different serotype, as described above. In contrast to the protection from challenge with Yam virus, mice immunized with vSHA1 failed to reduce the lung virus titre when compared with WR(HA<sup>-</sup>)-immunized mice (Table 1). However, immunization with vSNP1 was still effective in terms of reduction of the virus in the lung on day 7. Serum neutralizing antibody against Sic virus was not detected in any mice immunized with vSHA1, vSNP1 or WR(HA<sup>-</sup>).

To elucidate the mediator of protection from virus multiplication in the lung of mice immunized with vSHA1 or vSNP1, we analysed the ability of the recombinant vaccinia viruses vSHA1 and vSNP1 to prime an influenza-specific CTL response. BALB/c (H-2<sup>b</sup>) mice were immunized i.v. with 10<sup>7</sup> p.f.u. of the recombinant vaccinia virus. Two weeks later, their spleens were removed and restimulated in vitro with SW/HK-infected splenocytes from BALB/c mice. Effector cells were harvested after 5 days and assayed for specific cytotoxicity using a 51Cr release assay as described previously (Itamura et al., 1990b). Both the vSHA1 and vSNP1 primed subtype-specific CTL and strong cross-reactive CTL, respectively (Table 2).

Immunization with vSHA1 expressing influenza virus HA provided efficient protection from challenge infection in a subtype-specific manner. Challenge infection with the homologous virus was completely abolished by immunization with vSHA1, whereas immunization with vSHA1 did not protect mice from infection with heterologous H1N1 viruses although it reduced the lung virus titre. Amino acid sequence identities of HA (HA1 coding region) between SW/HK and Yam, and SW/HK and PR8 are 74.8~ and 80.4~ (Sugita et al., 1991; A. Endo, unpublished results). Similar subtype-specific protection by the recombinant vaccinia virus or fowlpox virus expressing avian influenza virus HA (H5) has been reported previously and the lowest sequence identity between vaccinated and challenge virus HA is still 85% (Chambers et al., 1988; Taylor et al., 1988).

Our results demonstrate the widest range of heterotypic protection. Since immunization with vSHA1 completely protected the lung from challenge infection with the homologous virus and produced high levels of virus-neutralizing antibody, the most plausible mediator of protection against the homologous virus is virus-neutralizing antibody. Undetectable levels of neutralizing antibody have been demonstrated to be valid for protection against challenge infection with influenza virus (Chambers et al., 1988; Andrew & Coupar, 1988;
Table 2. Ability of recombinant vaccinia viruses to prime influenza virus-specific CTL response

<table>
<thead>
<tr>
<th>Primed with</th>
<th>Specific cytotoxicity (%) to target cells infected with the following virus†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SW/HK (H1N1)</td>
</tr>
<tr>
<td>vSHA1</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>34.9</td>
</tr>
<tr>
<td>40</td>
<td>27.3</td>
</tr>
<tr>
<td>20</td>
<td>15.5</td>
</tr>
<tr>
<td>vSNP1</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>46.4</td>
</tr>
<tr>
<td>40</td>
<td>34.1</td>
</tr>
<tr>
<td>20</td>
<td>28.4</td>
</tr>
</tbody>
</table>

* E/T, Effector-to-target ratio.
† Cytotoxic activity of secondary in vitro stimulated splenocytes from BALB/c mice primed with vSHA1 or vSNP1 were measured against mastocytoma P815 (H-2a) cells infected with influenza viruses, SW/HK, Yam, Sic and B/Yamagata/16/88 (B/Yam) or uninfected (Mock) cells. Cytotoxicity was expressed as the percentage of specific 51Cr release from target cells.

Hunt et al., 1988). Therefore, virus-neutralizing antibody also may play an important role, even against heterologous H1N1 viruses, in the reduction of lung virus titre. In addition, CTLs also may contribute to clearance of heterologous H1N1 viruses from the host.

Immunization with vSNP1 expressing influenza virus NP reduced virus multiplication in the lungs of mice challenged with all type A influenza viruses examined, but it was less efficient although more cross-protective than immunization with vSHA1. Immunization with vSNP1 did not reduce the initial rate of replication in challenged mice and did not induce detectable neutralizing antibody, but it primed a cross-reactive CTL response against type A influenza viruses. These findings suggest that the protection conferred by vSNP1 is mediated by cross-reactive CTL. The above speculation is also supported by the following previous findings: (i) NP-specific antibody has no virus-neutralizing activity (Virelizier et al., 1979), (ii) CTLs aid in limiting viral replication and dissemination (Ennis et al., 1978), (iii) NP is a major target antigen for cross-reactive anti-influenza virus CTLs (Yewdell et al., 1985) and (iv) adoptive transfer of NP-specific CTL clones confers cross-protection from lethal challenge (Taylor & Askonas, 1986). Previous studies showed that mice vaccinated with the recombinant vaccinia virus expressing NP are poorly protected from mortality and morbidity (Andrew et al., 1987; Andrew & Coupar, 1988; Stitz et al., 1990). Reduction of lung virus titre by vSNP1 seems to be more efficient than that by the recombinant vaccinia virus reported previously (Andrew et al., 1987).

The difference in protective efficacy may be related to the insertion site in the vaccinia virus genome, the vaccinia virus promoter or the molecular species of the NP gene used for the construction of recombinant vaccinia virus. In our recombinant viruses, we used the HA gene locus as an insertion site, the NP gene of SW/HK virus and the new ATI–P7.5 hybrid promoter (containing the P7.5 early promoter element and the promoter of the gene encoding the major protein of cowpox virus A type inclusion body), which directed enhanced expression at both early and late phases of infection (S. Funahashi & H. Shida, unpublished results). In a previous study, the recombinant vaccinia virus was constructed to express the influenza NP gene of A/PR/8/34 (H1N1) virus under the control of the P7.5 promoter from the thymidine kinase (TK) gene locus (Andrew et al., 1987). Indeed, we observed the effect of insertion sites (vaccinia virus TK and HA gene loci) on the ability of recombinant vaccinia viruses to prime an influenza virus H3-specific CTL response (Itamura et al., 1990b). Recent studies also revealed that induction of CTL is profoundly affected by the molecular species expressed from recombinant vaccinia virus, by its temporal expression and its degradation (Coupar et al., 1986; Townsend et al., 1988).

Influenza viruses change antigenically with time in an unpredictable way; therefore, a vaccine able to elicit cross-reactive immunity would be of advantage. Although it has been suggested that NP is a candidate for vaccines promoting cross-reactive immunity, others have shown that immunization with the NP-recombinant virus alone is insufficient to protect against challenge (Andrew & Coupar, 1988; Stitz et al., 1990). Our data may however encourage further improvement of recombinant vaccinia virus expressing NP.

In mice infected with influenza virus, morbidity and mortality occur as immunopathological consequences rather than a direct effect of virus multiplication (Singer et al., 1972; Hurd & Heath, 1975; Sullivan et al., 1976). NP-recombinant virus limited virus propagation in the lung although it could not prevent infection and disease. Therefore, partially cross-protective NP-recombinant virus may be beneficial in the prevention of fatal pneumonia in man. Alteration of the vaccinia virus promoter and/or co-expression of lymphokine genes such as interleukin 2 or interferon-γ may increase the level of cross-reactive immunity obtainable by NP-recombinant vaccinia virus (Coupar et al., 1986; Yilma et al., 1987; Flexner et al., 1988; Davison & Moss, 1989).

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References


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