Characterization of antibody and cytotoxic T lymphocyte responses to human influenza virus H3 haemagglutinin expressed from the haemagglutinin locus of vaccinia virus

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Antibody and cytotoxic T lymphocyte (CTL) responses to the haemagglutinin (HA) of human H3N2 influenza virus were analysed, using recombinant vaccinia viruses containing the influenza HA gene inserted into the HA gene locus of vaccinia virus. The recombinant vaccinia viruses elicited a high haemagglutination inhibiting (HI) antibody response to the homologous influenza virus in mice. In addition, HI antibody generated by the recombinant vaccinia virus reacted with antigenic variants of human H3N2 influenza virus in a manner similar to that elicited by the HA vaccine. Mice with a high response to influenza virus HA vaccine were highly responsive to the HA expressed from the recombinant vaccinia virus, as measured by HI antibody production. The immunogenicity of the influenza virus HA expressed by the recombinant seems to be attributable to the intrinsic immunogenicity of the HA molecule. The recombinants primed mice for an influenza virus H3-specific CTL response and primed CTLs recognized the target cells in a subtype-specific manner. The results indicate that a recombinant vaccinia virus derived by the insertion of a foreign gene into its HA gene locus is a potent live vaccine not only for eliciting a high antibody response but also for priming a specific CTL response.

**Introduction**

Recombinant vaccinia viruses have been successfully used as live vaccines to protect a variety of animals against many infectious pathogens (reviewed in Moss & Flexner, 1987). Acquired immunity to a recombinant vaccinia virus limits the subsequent immune response to another (Rooney et al., 1988). Therefore, a single inoculation with a recombinant vaccinia virus expressing multiple protective antigens is desirable. For this purpose, several sites have been reported for the insertion of foreign genes into the vaccinia virus genome (Panicali & Paoletti, 1982; Mackett et al., 1984; Perkus et al., 1985, 1986, 1989) and a recombinant vaccinia virus expressing multiple protective antigens has been constructed (Perkus et al., 1985). The haemagglutinin (HA) gene is one of the insertion sites in the vaccinia virus genome and several recombinant vaccinia viruses have been constructed by using this insertion site, to express the envelope genes of human T cell leukaemia virus type 1 (Shida et al., 1987, 1988) and bovine leukaemia virus (Ohishi et al., 1988), and the HA genes of rinderpest virus (Tsukiyama et al., 1989) and influenza virus (Itamura et al., 1990). Although immunization with these recombinant vaccinia viruses could induce neutralizing antibodies against specific pathogens and protect the animals from challenging infections, cellular immune responses have not been characterized.

Previous studies showed that recombinant vaccinia viruses expressing H1, H2 and H3 HA genes of human influenza virus elicit haemagglutination inhibiting (HI) and virus-neutralizing antibodies, and protect animals from infection with homologous strains of influenza virus (Panicali et al., 1983; Smith et al., 1983; Jakeman et al., 1989). In addition, recombinant vaccinia viruses expressing the H1 and H2 HA genes induce a cytotoxic T lymphocyte (CTL) response (Bennink et al., 1984; Townsend et al., 1986), whereas a recombinant expressing the H3 HA gene was unable to do so (Yewdell & Hackett, 1989). The HA of human influenza virus has been antigenically drifting throughout their evolution. Although the protective efficacy against heterologous antigenic variants of influenza virus is one of the major concerns for human influenza vaccination, the antibody
response to variants elicited by the recombinant vaccinia viruses has not been adequately characterized, except for influenza B virus variants (Rota et al., 1987, 1989).

Protective immunity should be optimized in animals vaccinated with the recombinant vaccinia virus. As a first step to construct more efficient live vaccines, we have characterized the antibody response to antigenic variants elicited by recombinant vaccinia viruses expressing the H3 HA gene of human influenza virus (Itamura et al., 1990), and the priming activity of these recombinant viruses for a CTL response. This has been done to discover the immunological potency of the recombinant vaccinia virus with a foreign gene inserted into the HA gene locus and to examine the effect of the insertion configuration of a foreign gene on its immunogenicity.

Methods

Cells and viruses. RK13, CV-1 and L929 (H-2b) cells were grown at 37 °C in Eagle's MEM supplemented with 5% foetal calf serum (FCS). Mastocytoma P815 (H-2d) cells were grown in the peritoneal cavity of DBA/2 (H-2b) mice. Influenza A viruses, A/NIigata/102/81 (NG81) (H3N2), A/SW/Cambridge/39 (H1N1) and influenza B/Nagasaki/1/87 were grown in the allantoic cavity of 11-day-old embryonated chicken eggs. Formalin-fixed influenza viruses, A/Bangkok/1/79 (H3N2), A/Philippines/2/82 (H3N2), A/Yamagata/96/85 (H3N2) and A/Fu-

ngue/102/81 (NG81) has been used previously to con-
struct two recombinant vaccinia viruses, vR-1 and vR-2, which express the influenza virus H3 HA gene of NG81, were constructed previously (Itamura et al., 1990). Wild-type vaccinia virus (strain WR) and recombinant vaccinia viruses were grown in RK13 cells and purified essentially as described by Joklik (1962). P.f.u. titres were determined on RK13 cells.

Mice. Four-week-old female ddY, 6-week-old male BALB/c (H-2d), C3H/He (H-2b) and C57BL/6 (H-2b) specific pathogen-free mice were purchased from Japan SLC.

 Immunoprecipitation of influenza virus HA. Immunoprecipitation analysis was done as described previously (Itamura et al., 1990).

 Titration of HI antibody to influenza virus. Sera were examined against 4 haemagglutinating units (HAU) of each influenza virus following receptor-destroying enzyme (Takeda Pharmaceutical Co.) treatment and heat inactivation (56 °C for 30 min). Serial twofold dilutions of sera were prepared beginning at 1/32, and the HI titre was expressed as the reciprocal of the highest dilution causing complete inhibition of haemagglutination.

 Preparation of influenza virus HA vaccine. Influenza virus HA vaccine of NG81 (H3N2) was prepared according to Davenport et al. (1964). HA vaccine (0.5 ml) containing 100 chick cell-agglutinating (CCA) units was used for intraperitoneal immunization.

 In vitro generation of influenza virus-specific CTLs. BALB/c (H-2d) or C3H/He (H-2b) mice were inoculated intravenously with 106 p.f.u. of the wild-type or recombinant vaccinia virus. Two weeks later, their spleens were removed, and 5 x 107 splenocytes were mixed with 1 x 107 syngeneic, 2000 rads-irradiated spleen cells, infected with 200 HAU of influenza virus HG81 (H3N2), in 10 ml of complete medium (RPMI 1640, 5 x 10-5 M-2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin) supplemented with 15% FCS. Effector cells were harvested after 5 days of incubation at 37°C and assayed for specific cytotoxicity.

 Cytotoxicity assay. Cytotoxicity was measured by using a 51Cr release assay. Target cells were prepared by using P815 or L929 cells. The cells were infected with influenza virus at a multiplicity of about 120 HAU per 106 cells at 37°C for 30 min. After washing, cells (5 x 109) were incubated at 37°C for 4 h, and then labelled with 100 μCi of Na2-1CrO4 (200 μCi/ml, 10 to 35 mCi/mmol) at 37°C for 90 min. The labelled cells were washed three times, suspended in complete medium supplemented with 10% FCS, and 2 x 106 cells (100 μl) were dispensed to each well of a 96-well microplate. Effector cells in 200 μl were added at ratios of 20:1, 10:1, and 5:1, and the plate was centrifuged at 250 g for 5 min. After incubation at 37°C for 4 h, cell culture fluid was harvested with a Skatron Supernatant Collection System (Skatron) and its radioactivity was measured in a gamma scintillation counter. Spontaneous and maximum release were measured by addition of medium and 0-045 m-NH4OH, respectively, instead of effector cells. Each point was assayed in triplicate. The percent specific release of 51Cr was calculated as follows: ([experimental release – spontaneous release]/(maximum release – spontaneous release)) x 100. Spontaneous release was usually 20 to 30% of the maximum release.

 Depletion of effector cells generated in vitro. Effector cells were incubated at 4°C for 1 h with anti-Thyl.2 monoclonal antibody (Cedarlane Laboratories) diluted at 1:500, anti-Lyt2.2 (CD8) monoclonal antibody (Cedarlane Laboratories) at 1:20 or anti-asialoGM1 antibody (Wako) at 1:200. After washing twice, cells were incubated with 1:12 diluted Low-Tox rabbit complement (Cedarlane Laboratories) for 1 h at 37°C. Cells were then washed twice, suspended in complete medium supplemented with 10% FCS, and assayed for specific cytotoxicity.

 Results

 Characterization of influenza HA expressed by recombinant vaccinia viruses

 The H3 HA gene of human influenza virus A/Niigata/102/81 (NG81) has been used previously to construct two recombinant vaccinia viruses, vR-1 and vR-2, by insertion into the vaccinia virus HA gene locus (Itamura et al., 1990). Recombinant viruses vR-1 and vR-2 contain the influenza virus HA gene in the same orientation to the vaccinia virus P7.5 promoter, and this expression cassette is placed in the opposite (vR-1) or same (vR-2) direction to the flanking vaccinia virus HA gene promoter (Fig. 1).

 To examine the difference on the expression of influenza virus HA between two recombinant vaccinia viruses, CV-1 cells infected with vR-1 or vR-2 were labelled with [35S]methionine at 4 h post-infection for 3 h and immunoprecipitated with rabbit antisera specific for the H3 subtype of influenza virus (Fig. 2). A polypeptide of about 80K was specifically detected in both vR-1- and vR-2-infected cells. The molecular size of the expressed HA corresponds to glycosylated and uncleaved HA. Although in the preceding paper we found that the time course of HA activity was similar in
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**Fig. 1.** Structure of recombinant vaccinia virus constructs expressing influenza virus HA. $P_{HA}$, promoter of vaccinia virus HA gene; $P_{7.5}$, vaccinia virus P7.5 promoter; HA, influenza virus HA gene from A/Niigata/102/81 (H3N2).

**Fig. 2.** Expression of the H3 HA gene from human influenza virus by recombinant vaccinia viruses. CV-1 cells were infected with vR-1 (lanes 1 and 2) or vR-2 (lanes 3 and 4), and labelled with $[^{35}S]$methionine. The influenza HA polypeptide was then immunoprecipitated with the antiserum specific for H1 (lanes 1 and 3) or for H3 (lanes 2 and 4). Immunoprecipitates were analysed by electrophoresis in a 12% polyacrylamide gel containing SDS (Laemmli, 1970) and fluorographed. The positions of $M_r$ markers and the HA band (arrowhead) are indicated.

Both vR-1- and vR-2-infected cells during the late phase of infection (Itamura et al., 1990), enhanced expression of the HA was observed in vR-1-infected cells during the early phase after infection (Fig. 2). The biological activities and antigenicity of the expressed HA by vR-1 and vR-2 have been demonstrated to be similar to the authentic influenza virus HA (Itamura et al., 1990).

**Antibody response of mice to recombinant vaccinia viruses expressing the human influenza virus H3 subtype of HA**

First we examined the time course of the antibody response to recombinant vaccinia viruses expressing the influenza virus H3 HA gene. HI antibody titres to the homologous influenza virus NG81 (H3N2) were monitored in sera from ddY mice at various times after inoculation of vR-1 by two different routes (Fig. 3a). HI antibody was detected 1 week post-infection and had increased by 3 weeks. Similar responses were observed in mice inoculated intravenously and intraperitoneally. Both vR-1 and vR-2 elicited a similar antibody response (Fig. 3b). For comparison, influenza virus HA vaccine of NG81 was also administered to ddY mice intraperitoneally. The HI antibody titre increased with delayed kinetics (Fig. 3c).
Next we analysed antibody responses of various inbred mice to vR-1 and HA vaccine (Fig. 4). Three inbred mouse strains were vaccinated and showed relatively lower antibody responses than ddY mice. Although the level of antibody response to the HA vaccine was lower than that in response to vR-1, the pattern of antibody responses to vR-1 and the HA vaccine was similar among various mouse strains.

Finally, we characterized the reactivity of sera from ddY mice immunized with vR-1 or HA vaccine with antigenically drifted strains of H3N2 human influenza virus isolated from 1979 to 1985 (Fig. 5). The reactivity of sera from vR-1-immunized mice with the drift strains was similar to that of sera from HA vaccine-immunized mice and from ferrets infected with the influenza virus NG81 although the post-infection ferret serum reacted with the homologous strain to a greater extent than did sera from vR-1- and HA vaccine-immunized mice.

**Priming activity of recombinant vaccinia viruses for a CTL response.**

We examined the ability of the recombinant vaccinia viruses vR-1 and vR-2 to prime for a secondary CTL response. Splenocytes from BALB/c mice previously immunized with the recombinant vaccinia viruses were stimulated in vitro with autologous splenocytes infected with influenza virus NG81 (H3N2) and tested for their ability to lyse P815 cells infected with various influenza viruses. The effector cells primed with vR-1 or vR-2 were able efficiently to lyse cells infected with NG81 (H3N2), but failed to lyse cells infected with A/SW/Cambridge/39 (H1N1) or B/Nagasaki/1/87 (Fig. 6a, b). The specificity of vR-1 and vR-2 in priming for a cytotoxic response is shown by the failure of the parental vaccinia virus (WR) to stimulate anti-influenza virus-specific cytotoxic activity (Fig. 6c). The priming activity of vR-1 for a cytotoxic response was slightly higher than that of vR-2.

To examine whether this cytotoxic activity is due to CTL activity, we next characterized effector cells primed by vR-1. Splenocytes pretreated with anti-Thy1.2 or anti-CD8 monoclonal antibody plus complement failed to kill target cells infected with NG81 (Table 1). In contrast, pretreatment with anti-asialoGM1 antibody
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Fig. 6. The ability of recombinant vaccinia viruses to prime a CTL response to influenza virus. BALB/c mice were primed with vR-1 (a), vR-2 (b) or wild-type vaccinia virus (c). Effector cells stimulated in vitro were assayed for specific cytotoxicity against NG81 (H3N2) (O), A/SW/Cambridge/39 (H1N1) (●), B/Nagasaki/1/87 (□) and mock-infected target cells (■), respectively.

Table 1. Characterization of cytotoxic effector cells primed by the recombinant vaccinia virus vR-1

<table>
<thead>
<tr>
<th>Treatment of splenocytes†</th>
<th>Specific 51Cr release (%)*</th>
<th>NG81</th>
<th>Mock</th>
<th>NG81</th>
<th>Mock</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20:1</td>
<td>10:1</td>
<td>20:1</td>
<td>10:1</td>
</tr>
<tr>
<td>Anti-Thy1.2 + C'</td>
<td></td>
<td>4.6</td>
<td>3.2</td>
<td>4.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Anti-CD8 + C'</td>
<td></td>
<td>4.9</td>
<td>3.6</td>
<td>4.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Anti-asialoGM1 + C' C'</td>
<td></td>
<td>24.5</td>
<td>15.9</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.4</td>
<td>17.9</td>
<td>3.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* Cytotoxic activity of effector cells was expressed as percentage of specific 51Cr release of NG81- or mock-infected P815 cells at given effector:target ratios.
† Secondary in vitro stimulated splenocytes were treated with antisera plus complement (C').

Table 2. MHC-restricted cytotoxic activity of effector cells primed by the recombinant vaccinia virus vR-1

<table>
<thead>
<tr>
<th>Mouse strain†</th>
<th>Specific 51Cr release (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P815 (H-2°)</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
</tr>
<tr>
<td>BALB/c (H-2°)</td>
<td>36.7</td>
</tr>
<tr>
<td>C3H/He (H-2°)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Cytotoxic activity of effector cells was expressed as percentage of specific 51Cr release of NG81- or mock-infected two target cells at given effector:target ratios.
† Effector cells primed with vR-1 were generated from BALB/c (H-2°) or C3H/He (H-2°) mice.

Discussion

In this study, we have characterized the immunogenicity of recombinant vaccinia viruses expressing the H3 HA gene of human influenza virus, the construction of which is based on the insertion into the HA gene locus of vaccinia virus. The results described here indicate the high immunogenicity of the recombinant vaccinia viruses to elicit an antibody response and prime for a specific CTL response. Many recombinant vaccinia viruses have been constructed previously by inserting the foreign gene into the thymidine kinase gene or other sites, and have been shown to elicit a high antibody response and to prime and stimulate a specific CTL response (reviewed in Moss & Flexner, 1987). Although several recombinants constructed using the HA gene locus of vaccinia virus as an insertion site have been demonstrated to induce neutralizing antibody and to protect vaccinated animals from subsequent challenge infection (Shida et al., 1987, 1988; Ohishi et al., 1988; Tsukiyama et al., 1989), this is the first characterization of priming ability of the recombinant vaccinia virus, with a foreign gene inserted into the HA gene, for a CTL response.

The recombinant vaccinia virus could generate an HI antibody response to antigenic variants of human H3N2 influenza virus, which was similar to the HI antibody response elicited by the influenza virus vaccine from the same strain as that providing the HA gene for the recombinant vaccinia virus. In addition, antibody responses of mouse strains to the recombinant vaccinia virus expressing the influenza H3 HA gene paralleled those to the HA vaccine. Wood et al. (1989) speculated on the possible difference of immune responses elicited by inactivated influenza vaccine and influenza virus HA expressed from the vaccinia virus vector. However, our
results indicate that the antibody response to the influenza virus HA expressed by the recombinant vaccinia virus reflects the immunogenicity of the HA molecule.

We demonstrated priming ability of the recombinant vaccinia viruses for an influenza virus-specific CTL response. The cytotoxic effector cells primed by the recombinant vaccinia virus were characterized as conventional CD8+ CTLs, based on the following criteria: target specificity of the cytotoxic activity, the surface markers of effector cells and MHC-restricted recognition of target cells. CTLs primed with the recombinant vaccinia virus expressing the H3 HA gene of human influenza virus could recognize the target cells in a subtype-specific manner. This observation is similar to the specificity of CTL recognition primed by the recombinant vaccinia viruses expressed H1 and H2 HA genes of human influenza virus (Bennink et al., 1984; Townsend et al., 1986). Curiously it has not been possible to demonstrate an H3-specific CTL response in many mouse strains primed with a recombinant vaccinia virus expressing the H3 HA gene (Yewdell & Hackett, 1989).

To our knowledge, this is the first demonstration of a priming ability of recombinant vaccinia viruses expressing the H3 HA gene of influenza virus for a specific CTL response. This discrepancy may be explained as follows: either the influenza H3 HA genes used for construction of the recombinant vaccinia virus are different, or the influenza H3 HA genes have been inserted at different sites in the vaccinia virus genome. Recent studies revealed that the induction of CTLs and the presentation to CTLs are profoundly affected by temporal expression of a foreign gene inserted in vaccinia virus and degradation efficacy of the expressed protein (Coupar et al., 1986; Townsend et al., 1988). We also found different priming abilities for the influenza virus-specific CTL response between two recombinant vaccinia viruses which differ in the orientation of insertion of the H3 HA gene under the control of the 7.5K promoter with respect to the flanking vaccinia virus HA gene (Fig. 1 and Fig. 6). This seems to be related to the different levels of early expression of the influenza virus H3 HA gene in infected cells (Fig. 2), although the expression kinetics during the late phase of infection in these recombinant virus-infected cells is similar (Itamura et al., 1990). Further studies are required to clarify the molecular basis of the different abilities of our recombinant vaccinia viruses to prime a CTL response.

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References


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