Protective efficacy in mice of post-exposure vaccination with vaccinia virus recombinant expressing either rabies virus glycoprotein or nucleoprotein

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Mice vaccinated intraperitoneally (i.p.) with 107 p.f.u. of a vaccinia virus recombinant expressing either the glycoprotein (rVac-G) or nucleoprotein (rVac-N) of rabies virus 3 weeks before challenge were protected against peripheral lethal infection. Similarly, by post-exposure vaccination in which mice were first infected with rabies virus and subsequently vaccinated i.p. with the recombinant, rVac-G conferred protection when given immediately following infection and up to 24 h after infection. Prior treatment of those mice with anti-CD8 monoclonal antibodies (MAb) did not significantly affect the outcome of the infection. In contrast, rVac-N failed to confer protection even with higher doses (108 p.f.u.) of the virus or even when administered by the intradermal route. Anti-nucleoprotein antibody production by these mice was not suppressed by prior rabies virus infection and the levels and the time of antibody production were similar to those of anti-glycoprotein antibody production in mice vaccinated with rVac-G after rabies virus infection. The cytotoxic T lymphocyte response was also not down-regulated by rabies virus in the mice that were given rVac-N. Possible mechanism(s) for the ineffectiveness of rVac-N by post-exposure vaccination in contrast to pre-exposure vaccination was discussed.

Introduction

Rabies virus is a member of the Rhabdoviridae consisting of a single negative-strand RNA genome and five virus-encoded proteins. Among these, nucleoprotein (N), phosphoprotein and RNA transcriptase are tightly associated with genomic RNA and form a transcriptionally active nucleocapsid (RNP). The two other proteins are membrane-associated: the externally oriented transmembrane glycoprotein (G) and the peripheral matrix protein.

Rabies virus G protein is thought to be the major antigen responsible for the induction of virus-neutralizing antibodies and for conferring protection against lethal rabies infection (Cox et al., 1977; Lafon et al., 1983; Wunner et al., 1983; Bunschoten et al., 1989). A cytotoxic T cell clone recognizing the G protein has also been demonstrated to play a role in protection (Kawano et al., 1990). Experiments with recombinant viruses expressing the G protein have confirmed the importance of this protein in the induction of protective immunity (Kieny et al., 1984; Wiktor et al., 1984; Esposito et al., 1987; Brochier et al., 1990; Thomas et al., 1990; Prevec et al., 1990).

Purified rabies RNP, which does not induce virus-neutralizing antibodies, was also shown to confer protective immunity (Dietzschold et al., 1987). Further studies have demonstrated that protection can be achieved by pre-exposure vaccination of animals with the N protein alone, although the underlying mechanism(s) is unclear (Sumner et al., 1991; Lodmell et al., 1991; Fu et al., 1991; Fekadu et al., 1992). More recently, we have confirmed the protective ability of the N protein and demonstrated that a considerable part of such protection achieved by pre-exposure vaccination can be ascribed to the intact anti-N antibody (Takita-Sonoda et al., 1993).

In the present study, we examined the protective efficacy of vaccinia virus recombinants expressing either rabies virus G (rVac-G) or N (rVac-N) protein following post-exposure administration to mice. Post-exposure vaccination is the usual treatment for humans that are infected with the virus.

Methods

**Virus and cell cultures.** The 1088 strain of rabies virus at the second passage level in suckling mouse brain (Mifune et al., 1980) was used as a challenge virus. Vaccinia virus recombinants that express the G protein (rVac-G) or N protein (rVac-N) of the Challenge Virus Standard (CVS) strain of rabies virus were established for our previous study (Takita-Sonoda et al., 1993) and grown in rabbit kidney (RK13) cells.

Murine neuroblastoma (NA) cells, syngeneic to A/J mice, NA cells persistently infected with the HEP-Flury strain of rabies virus
Table 1. Protective efficacy in mice of post-exposure injection of vaccinia virus recombinants expressing the rabies virus G or N protein

<table>
<thead>
<tr>
<th>Rabies virus infective dose (MFPLD₉₀)</th>
<th>Time of injection in relation to rabies virus infection</th>
<th>Injected with (p.f.u.)</th>
<th>Route</th>
<th>No. of survivors/ no. of mice</th>
<th>Mean day of death ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>3 weeks and 1 week before</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>10/10</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>Immediately after</td>
<td>rVac-G (5 x 1⁰⁶)</td>
<td>i.p.</td>
<td>10/10</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>Immediately after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>15/15</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>6 h after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>4/5</td>
<td>17</td>
</tr>
<tr>
<td>20</td>
<td>12 h after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>7/8</td>
<td>17</td>
</tr>
<tr>
<td>20</td>
<td>24 h after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>7/8</td>
<td>17</td>
</tr>
<tr>
<td>20</td>
<td>48 h after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>2/8</td>
<td>12±5 ± 0.5</td>
</tr>
<tr>
<td>160</td>
<td>Immediately after</td>
<td>rVac-G (1⁰)</td>
<td>i.p.</td>
<td>5/6</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>3 weeks and 1 week before</td>
<td>rVac-N (1⁰)</td>
<td>i.p.</td>
<td>12/13</td>
<td>23</td>
</tr>
<tr>
<td>20</td>
<td>Immediately after</td>
<td>rVac-N (1⁰)</td>
<td>i.p.</td>
<td>1/15</td>
<td>12±8 ± 1/4</td>
</tr>
<tr>
<td>20</td>
<td>Immediately after</td>
<td>rVac-N (2 x 1⁰⁸) intradermal</td>
<td>i.p.</td>
<td>1/10</td>
<td>14±4 ± 1/3</td>
</tr>
<tr>
<td>20</td>
<td>Immediately after</td>
<td>rVac-N (1⁰)</td>
<td>i.p.</td>
<td>2/12</td>
<td>13±8 ± 1/9</td>
</tr>
<tr>
<td>20</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>1/10</td>
<td>13±5 ± 1/9</td>
</tr>
</tbody>
</table>

(NA-HEP) (Cho et al., 1987) and NA cells expressing only the G (NA-pAX-G) or N (NA-pAX-N) protein of rabies virus, generated from transfectants of the respective cDNA with the aid of eukaryotic expression vector pAX-91 (K. Mannen & K. Mifune, unpublished), were grown in Dulbecco’s modified MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. These cells were used as target cells for cytotoxic T lymphocytes (CTL) in the ⁵¹Cr release assay.

Mice and vaccination of mice. Inbred A/J mice purchased from an animal laboratory were used. For pre-exposure vaccination experiments, 4-week-old mice were injected intraperitoneally (i.p.) on days 0 and 14 with 10⁷ p.f.u. of the recombinant viruses. Control mice received equal doses of vaccinia virus recombinant vSC8 (Chakrabarti et al., 1985) that contained no rabies virus sequences. On day 21, the mice were challenged by footpad injection with 0.02 ml (20 MFPLD₀, where MFPLD₀ represents mouse footpad 50% lethal dose) of the 1088 strain.

In post-exposure vaccination experiments, 7-week-old mice infected in the footpad with 20 MFPLD₀ (160 MFPLD₀ in one experiment) of rabies virus were injected i.p. (intradermally in one experiment) with various doses (from 5 x 1⁰⁵ to 10⁶ p.f.u.) of recombinant viruses immediately, 6 h, 12 h, 24 h and 48 h after rabies virus infection. Mice were observed for 4 weeks and deaths were recorded daily.

Determination of anti-G and anti-N antibody titres. An indirect ELISA was performed for detecting anti-G and anti-N antibody against rabies virus. Each well of a microtitre plate (Nunc) was coated with 100 µl of the G and N antigens diluted to 0.4 µg/ml in coating buffer. The G protein used as the antigen was derived from soluble G protein that was truncated just external to the transmembrane domain of native G protein from the Nishigahara strain and secreted into culture medium from SF21 insect cells by the baculovirus expression system (K. Mannen & K. Mifune, unpublished). Nucleocapsid was extracted and purified from BHK-21 cells infected with the same virus by CsCl isopycnic density gradient centrifugation as described by Sokol et al. (1969) and was then used as the N antigen. After blocking and washing, the plates were incubated for 2 h at 37°C with 100 µl per well of test serum samples. After washing, 100 µl of anti-mouse peroxidase-labelled IgG (Cappel) that had been diluted to 1:2000 was added before a further incubation for 1 h at 37°C. After the last washing, the substrate solution was added to each well, and the A₄₉₂ of the reaction mixture was measured with a microplate reader. Antibody titre was expressed as the reciprocal of the maximum serum dilution showing an A₄₉₂ value of more than twice that of control serum.

Cytotoxicity assay. Target cells (NA-pAX-G and NA-pAX-N) were incubated with 10 mm-sodium butyrate and ¹⁵CrNa₂O₄ at 100 µCi/ml overnight, dispersed with trypsin and washed three times. The cells were then plated in duplicate onto flat-bottom microwell plates (Nunc) at 2 x 1⁷ cells per well suspended in 0.1 ml of culture medium. Murine spleen cells, suspended in 0.1 ml of Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated FBS, 5 x 10⁻³ M-2-mercaptoethanol, 1 mM L-glutamine and antibiotics, were added to the wells to yield a range of effector:target ratios. The assay was carried out in duplicate and completed after incubation at 37°C for 6 h. Specific lysis (%) was calculated as [(⁵¹Cr release with effector - ⁵¹Cr release in medium)/(⁵¹Cr release with 1% Triton X-100 - ⁵¹Cr release in medium)] x 100. The background level of this assay system was usually below 5% of total ⁵¹Cr release with 1% Triton X-100 (H. Fujii, K. Mannen, Y. Takita, M.S. Cruz-Abrencia, K. Hirai, A. Nishizono & K. Mifune, unpublished).

Results

Protective efficacy against rabies of post-exposure vaccination

Control experiments that were conducted to determine the protective efficacy of rVac-G and rVac-N in pre-exposure vaccination showed that 100% of the mice injected i.p. with rVac-G and 92% of mice given rVac-N (i.e. one animal succumbed but still had a prolonged life span) survived the challenge. Similarly, in mice given rVac-G immediately following infection and up to 24 h after rabies virus infection, 80% or more were protected. The survival rate, however, decreased significantly when the recombinant virus was administered 48 h after infection. In addition, mice infected with higher doses of virus (160 MFPLD₀) and vaccinated immediately with
Table 2. Effect of anti-CD8 MAb on the outcome of mice given rVac-G after exposure to rabies virus*  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of survivors/ no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies virus + rVac-G</td>
<td>8/9</td>
</tr>
<tr>
<td>Anti-CD8 + rabies virus + rVac-G</td>
<td>12/14</td>
</tr>
<tr>
<td>Rabies virus only</td>
<td>0/8</td>
</tr>
</tbody>
</table>

* Mice that were injected i.p. with 0.2 ml of rat anti-mouse Lyt 2 MAb three times on days -1, 0 and 4 were infected with 10 MFPLD₅₀ of rabies virus and then injected i.p. with 10⁷ p.f.u. of rVac-G at 6 h after rabies virus infection.

Effect of anti-CD8 MAb on resistance in rVac-G-vaccinated mice

In order to determine the role of CD8⁺ T cells in the protection arising from post-exposure vaccination of rVac-G, mice that had been injected i.p. with 0.2 ml of rat anti-mouse Lyt 2 monoclonal antibody (MAb) (Caltag Laboratory) three times, on days -1, 0 and 4, were infected with 10 MFPLD₅₀ of the rabies virus strain 1088 and inoculated i.p. with 10⁷ p.f.u. of rVac-G 6 h after infection. In preliminary experiments, this regime of anti-CD8 administration was shown to deplete CD8⁺ T cells in the spleen more than 91%, 85% and 80% on days 5, 12 and 22 after infection, respectively. In addition, the CTL response specific for the G protein was completely inhibited by this treatment (Fig. 2b). In this CTL assay system, the cytotoxic activity of the spleen cells obtained from mice immunized with either rVac-G or rVac-N was completely prevented by prior treatment of the spleen cells with anti-CD8 antibodies plus complement but not with anti-CD4 antibodies plus complement (data not shown). The results (Table 2) showed that such depletion of CD8⁺ T cells had no significant effect on the outcome of the virus challenge, suggesting that generation of CD8⁺ CTLs is not significantly involved in the protection achieved by post-exposure vaccination with rVac-G.

Antibody response after post-exposure vaccination

In an attempt to elucidate the underlying mechanism by which protection can be achieved by post-exposure vaccination with rVac-G but not rVac-N, antibody responses against the G and N proteins were first examined in mice vaccinated with virus recombinants immediately after infection. Four mice from each group were sacrificed on days 4 and 8 and the serum samples were collected by cardiac puncture in order to determine anti-G and anti-N antibody levels by ELISA. Anti-G antibody production was not suppressed by prior rabies virus infection and the kinetics and magnitude of
antibody production were similar to those of anti-G antibody production in mice vaccinated with rVac-G after rabies virus infection (Fig. 1).

**CTL response after post-exposure vaccination**

It is known that resistance to rabies virus infection is mediated by both humoral and cell-mediated immunity (Sikes et al., 1971) and that the protective effect of post-exposure treatment is achieved only by vaccine and not by anti-rabies virus-neutralizing antibodies (Sikes et al., 1971; Baer & Yager, 1977; Mifune et al., 1980; Smith, 1981), although a recent study has demonstrated that an anti-G MAb that has an inhibitory effect on virus spread from cell to cell and on virus RNA transcription can afford protection against rabies in animals by post-exposure treatment (Dietzschold et al., 1992). Therefore, we next examined the CTL response in these mice although no evidence for an involvement of CTLs has been demonstrated in the protective effect induced by rVac-N in pre-exposure vaccination. It is possible that induction of a cell-mediated immune response by rVac-N is suppressed by prior virus infection, since street rabies virus infection has been shown to induce a severe suppression of cell-mediated immunity (Wiktor et al., 1977; Hirai et al., 1992). For this reason, overall CTL responses, as well as the CTL response to the two rabies virus proteins in mice given 10⁷ p.f.u. of either rVac-G or rVac-N immediately after infection with 20 MFPLD₉₀ of rabies virus, were examined using NA-HEP cells, pAX-G and pAX-N cells as target cells. The results (Fig. 2) showed that in mice given rVac-N a CTL response to the N protein and other viral proteins is not down-regulated and strongly resembles the CTL response in control mice given rVac-N alone. A similar effect occurred in the CTL response to the G protein in mice given rVac-G.

**Discussion**

The present study demonstrates that rVac-G is effective in conferring protection against rabies when given immediately and up to 24 h following infection, whereas rVac-N fails to confer protection even with higher doses of the vaccine virus. Infected mice were protected by only one dose of 10⁷ p.f.u. of rVac-G that was given considerably later following exposure to rabies virus indicating that the success of protection declines with increasing time between the exposure to rabies virus and beginning of vaccination.

Anti-G-CD8⁺ CTLs have been shown to play a protective role against murine rabies in our previous study (Kawano et al., 1990). This was a model for passive protection wherein both humoral and cell-mediated immune responses were inhibited. In this model, infection of mice with an attenuated strain of rabies virus became lethal upon treatment with cyclophosphamide. In the present study, however, mice given rVac-G after rabies virus infection were resistant to virus challenge even after depletion of CD8⁺ T cells. This finding is in agreement with the observations by Perry & Lodmell (1991). This might suggest that although anti-G CTLs might confer protection especially when antibody production is inhibited, the protection achieved by post-exposure...
vaccination with rVac-G is mediated by anti-G virus-neutralizing antibodies or by CD4+ T cell-mediated defence mechanisms.

The lack of efficacy in protection of rVac-N during post-exposure vaccination was in sharp contrast to the marked efficacy of rVac-N following pre-exposure vaccination. Observations that animals vaccinated in pre-exposure with recombinant vaccinia virus expressing the N protein or with purified N protein are resistant to subsequent challenge have accumulated (Dietzschold et al., 1987; Sumner et al., 1991; Lodmell et al., 1991; Fu et al., 1991; Fekadu et al., 1992; Takita-Sonoda et al., 1993). However, very little is known about the mechanism(s) of protection. It has been proposed that an efficient production of virus-neutralizing antibodies or anti-viral cytokines or both from CD4+ T cells in the animals primed with the N protein contributes to the protection (Dietzschold et al., 1987; Ertl et al., 1989; Fu et al., 1991). However, we were unable to detect anti-G antibody in surviving mice that had been vaccinated with rVac-N and challenged with street rabies virus (Takita-Sonoda et al., 1993), similar to observations made by Lodmell et al. (1991). More recently, it has been reported that anti-N antibodies are produced in animals; these have no virus-neutralizing activities but afford protection, although the mechanism(s) involved still requires further elucidation (Takita-Sonoda et al., 1993; Lodmell et al., 1993).

The present study attempted to delineate the mechanism(s) responsible for the lack of efficacy in protection of rVac-N given to mice after rabies virus infection. The kinetics and magnitude of anti-N antibody production in these mice were not significantly different from those of anti-G antibody in mice given rVac-G after infection. In addition, the level of CTL response to the N protein was very similar to that found in mice given rVac-N alone, suggesting that the anti-N CTL response is not significantly involved in the mechanism(s) of protection. In regard to the protective ability of anti-N antibodies, the most recent study by Lodmell et al. (1993) has demonstrated that sera from mice vaccinated in pre-exposure with rVac-N were not as effective in inhibiting rabies virus replication in vitro as sera from animals vaccinated with rVac-G. In their experiments, the inhibitory activity of anti-N serum was markedly reduced by dilution, whereas similar dilutions of anti-G serum had no effect on its ability to neutralize viral infectivity. Our preliminary data showed that more than 10 times the amount of anti-N antibody as that of anti-G antibody is required to achieve the same protection although precise values are difficult to evaluate when there are limited amounts of MAbs.

The lack of effective protection following post-exposure vaccination with rVac-N can be explained by insufficient production of anti-N antibody which inhibits virus spread into the central nervous system. The possibility that the failure may also be due to lower production of cytokines such as interferon from CD4+ T cells, as compared with the production in animals with pre-exposure vaccination, cannot be excluded, since a great difference in the population of N-primed CD4+ T cells would be expected between animals given pre-exposure and post-exposure vaccination with rVac-N.

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