Mutant forms of the F protein of human respiratory syncytial (RS) virus induce a cytotoxic T lymphocyte response but not a neutralizing antibody response and only transient resistance to RS virus infection

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Vaccinia virus (vv) recombinants expressing either wild-type (VA-F) or mutant forms (VA-FT, VA-FR47, VA-FS1 to VA-FS6) of the fusion (F) protein of respiratory syncytial (RS) virus were examined for their ability to elicit antibody, cytotoxic T lymphocytes (CTL) and protection against RS virus infection in BALB/c mice. Cells infected with the VA-F and VA-FT recombinants expressed the F protein on their surface and mice vaccinated with these recombinants developed RS virus neutralizing antibodies. The VA-FR47 recombinant expressed a mutant form of the F protein (with six amino acid changes from the wild-type) in which both proteolytic processing of the F₀ precursor and its transport to the cell surface were inhibited. These mutants induced transient protection against RS virus infection although they did not induce RS virus neutralizing antibodies, or antibodies detectable by ELISA. All the vv recombinants were able to induce an RS virus-specific, MHC class I restricted CTL response. Vaccination of mice with a second set of vv recombinants expressing mutant forms of the F protein showed that the replacement Phe to Ser at amino acid 237 either alone or in combination with others abolished the neutralizing antibody response but did not affect priming of CTLs. These results demonstrate that long-term protection against RS virus infection in mice vaccinated with recombinant vv expressing the F protein is more dependent upon the induction of an antibody rather than a CTL response.

Introduction

Human respiratory syncytial (RS) virus is a pneumovirus of the family Paramyxoviridae and is a major cause of lower respiratory tract infections in infants and young children (Stott & Taylor, 1985; McIntosh & Chanock, 1990). The pathogenesis of the disease is poorly understood, reinfection is common and there is currently no effective vaccine available (Pringle, 1987; Kimman & Westenbrink, 1990). In an early vaccine trial, children exposed to natural RS virus infection, following administration of a formalin-inactivated vaccine, exhibited exacerbated disease (Fulginiti et al., 1969; Kim et al., 1969). Enhanced pathology has also been observed in cotton rats and mice inoculated with formalin-inactivated virus, exhibited exacerbated disease (Fulginiti et al., 1969; Kim et al., 1969). Enhanced pathology has also been observed in cotton rats and mice inoculated with formalin-inactivated virus (Prince et al., 1986; Vaux-Peretz & Meignier, 1990) and in mice vaccinated with vaccinia virus (vv) recombinants expressing individual RS virus proteins (Stott et al., 1987; Openshaw et al., 1992). The enhanced pathology seen in vaccine recipients may involve Th2 responses as depletion of either CD4⁺ T cells or of both interleukin-4 (IL-4) and IL-10 abrogated the pathology observed in mice vaccinated with formalin-inactivated virus (Connors et al., 1992b, 1994). Further, passive transfer of RS virus-specific CD4⁺ or CD8⁺ T cells cleared virus from the lungs of infected mice but augmented lung pathology (Cannon et al., 1988; Alwan et al., 1992, 1994). In contrast, passively transferred antibodies, which reduce virus titres in the lungs, do not exacerbate pathology in RS virus-infected mice or cotton rats (Taylor et al., 1984, 1992; Walsh et al., 1984). These findings suggest that a successful vaccine should stimulate a long-lasting antibody response, but it is not clear if it is also important to stimulate a cytotoxic T lymphocyte (CTL) response.

The F glycoprotein of RS virus is the major cross-protective antigen between strains (Stott et al., 1987; Olmsted et al., 1986) and is expressed on the surface of infected cells. The F protein is synthesized as a F₀ precursor (69 kDa) and is co-translationally N-glycosylated and post-translationally cleaved into two subunits, F₁ (49 kDa) and F₂ (20 kDa), which are linked
Neutralizing and fusion-inhibiting MAbs to the F protein are mediated by neutralizing antibodies. The results support the view that long-lasting protection against RS virus challenge in mice induced by the F protein is mediated by neutralizing antibodies.

### Methods

**Viruses.** The human RS viruses of the Long strain, the A2 strain and the escape mutant R47F/4, selected with MAb 47F (Garcia-Barreno et al., 1989) were grown in HEp-2 monolayers and purified from culture supernatants as described previously (Garcia-Barreno et al., 1988). Recombinant vv expressing native F protein (VA-F) from the Long strain RS virus and mutant forms of the F protein in which expression at the surface of infected cells was inhibited, and vv containing plasmid pSC11 without an insert for use as a recombinant control (VA-5C), were produced and characterized (López et al., 1996). The amino acid changes in the F protein of the mutants and their effect on cell surface expression are summarized in Table 1. The recombinant vv were grown in HEp-2 cells by inoculation with 5 p.f.u./cell and incubation at 37°C until a cytopathic effect was observed in greater than 75% of the cells (usually 48 h). The cells were then scraped into the medium, pelleted and resuspended in 10 mm-Tris–HCl pH 8.8. The cell suspension was sonicated for 2 min and centrifuged at 1000 g for 5 min. The supernatant was removed and the pellet resuspended as before and the process repeated. The supernatants were pooled and stored at -70°C.

**Mice.** Six-week-old specific-pathogen-free BALB/c female mice (H-2b), obtained from Charles Rivers, were inoculated i.p. with 1 × 10⁶ p.f.u. of recombinant vv. Serum samples were obtained from the mice immediately before i.n. challenge with approximately 10⁵ p.f.u. of the A2 strain of RS virus. Five days after challenge, groups of five mice were killed and the RS virus titre in lung homogenates was determined by plaque assay on secondary calf kidney cells (Taylor et al., 1984). Mean titres of virus from groups were compared by unpaired Student's t-test. Serum was obtained from further groups of five mice killed 7 days after challenge; the lungs from these mice were fixed in formal-sublimate and histological sections were stained with haematoxylin and cosin as described previously (Taylor et al., 1984). Cells were recovered from lung washings according to the method of Denny et al. (1972), but using 1 ml of PBS.

### Table 1. Characterization of the antibody response induced in mice by recombinant vv expressing mutant forms of the F protein of RS virus

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Virus</th>
<th>Amino acid changes</th>
<th>Intact F protein on cell surface</th>
<th>ELISA titre*</th>
<th>Reactivity in Western blot†</th>
<th>Neutralization titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VA-5C</td>
<td></td>
<td></td>
<td>50 &lt; 2</td>
<td>ND</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td>VA-F</td>
<td></td>
<td>+</td>
<td>4-1 &lt; 2</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>VA-FT</td>
<td>262</td>
<td>+</td>
<td>4-2 &lt; 4</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>VA-FR47</td>
<td>67, 120, 223, 237, 262, 442</td>
<td>-</td>
<td>4-3 &lt; 2</td>
<td>ND</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>2</td>
<td>VA-F</td>
<td></td>
<td></td>
<td>2-5 &lt; 2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS1</td>
<td>223</td>
<td>+</td>
<td>2-7 &lt; 2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS2</td>
<td>237</td>
<td>-</td>
<td>3-0 &lt; 1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS3</td>
<td>223, 237</td>
<td></td>
<td>2-8 &lt; 1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS4</td>
<td>223, 262</td>
<td>+</td>
<td>3-1 &lt; 2.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS5</td>
<td>237, 262</td>
<td></td>
<td>3-0 &lt; 1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS6</td>
<td>223, 237, 262</td>
<td>-</td>
<td>3-0 &lt; 1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* log₁₀ titre of antibody to either extracts of VA-5C infected cells (VACC) or immunoaffinity purified F protein (F).
† Reactivity of mouse sera with the F1 subunit.
‡ log₁₀ titre that inhibited cytopathic effect in a RS virus microneutralization test.

ND, Not determined.

by disulphide bonds (McIntosh & Chanock, 1990). Neutralizing and fusion-inhibiting MAbs to the F protein not only protect against RS virus infection in mice, cotton rats and calves (see Taylor, 1994; Walsh et al., 1984) but can clear an established infection (Tempest et al., 1991; see Taylor, 1994). In addition, the F protein is a target for both human and murine CTL (Pemberton et al., 1987; Nicholas et al., 1990; Connors et al., 1991; Cherrie et al., 1992) and Th responses (Openshaw et al., 1988; Alwan et al., 1993). Vaccination of mice with recombinant vv expressing the F protein alone induces neutralizing antibody, a Th1 and a CTL response. Adoptive transfer of F protein-specific T cell lines reduced the levels of virus in the lungs of RS virus-infected mice (Alwan et al., 1994). However, the role of antibodies and T cell responses in the protection mediated by the F protein is not clear. Immunization of mice with vv recombinants expressing the F protein protected against RS virus challenge following depletion of CD4⁺ and CD8⁺ T cells prior to challenge (Connors et al., 1992a), suggesting that antibodies to the F protein were able to protect against RS virus challenge independent of a T cell response. However, resistance in vaccinated and depleted mice may have been mediated locally by residual levels of T cells or, since the vv recombinants were administered both intraperitoneally (i.p.) and intranasally (i.n.), it is possible that mucosal IgA antibodies mediated protection in depleted animals (Connors et al., 1992a). In this study, recombinant vv expressing F genes that contain mutations which result in defective maturation of the F₀ precursor, so that the native protein is not expressed on the surface of the infected cell (López et al., 1996), have been examined for their ability to elicit CTLs, induce neutralizing antibodies and protect against RS virus challenge in BALB/c mice.
Immune responses to RS virus F protein mutants

Fig. 1. Cytotoxic activity of splenocytes from mice inoculated with RS virus, VA-F, VA-FT or VA-FR47, tested at decreasing effector:target (E:T) ratios on uninfected BALB/c fibroblasts (○), BCH4 cells (□), uninfected L-929 cells (△) or RS virus-infected L-929 cells (●).

Antibody assays. The presence of antibodies to RS virus and vv recombinants was determined by ELISA, as described previously (Taylor et al., 1992). Micro-neutralization tests were carried out by incubating 1–5 × 10^4 p.f.u. of the Long strain of RS virus with twofold serial dilutions of sera for 60 min at 37 °C. The virus antibody mixtures were then used to infect HEp-2 monolayers grown in 96-well tissue culture plates. After 3 days at 37 °C, monolayers were fixed with 10% formaldehyde and stained with crystal violet. Neutralization titres were estimated from the highest dilution of sera that inhibited the destruction of cell monolayers, compared with an antiserum control.

Western immunoblotting was carried out using cell extracts that were separated into individual proteins by SDS-PAGE, electro-transferred to nitrocellulose paper (Towbin et al., 1979) and blocked overnight with 5% non-fat dried milk in PBS at room temperature. The paper was cut into strips and incubated for 2 h with 1:100 dilutions of the individual mouse sera diluted in blocking buffer, followed by incubation with biotinylated anti-mouse Ig, horseradish peroxidase-streptavidin–peroxidase and 4-chloro-1-naphthol as substrate.

Generation of secondary CTLs in vitro. Spleen lymphocytes from mice immunized with vv recombinants a minimum of 4 weeks earlier were restimulated in vitro with RS virus-infected splenocytes. Thus, spleen lymphocytes from uninfected mice were pelleted and incubated in 1 ml of medium containing 0.4 p.f.u./cell of the A2 strain of the virus for 90 min at 37 °C. Spleen cells from vaccinated mice were purified using a Histopaque gradient (density 1.083, Sigma), washed and incubated with 3 × 10^6 RS virus-infected control splenocytes, at an effector:stimulator ratio of 5:1, for 5 days in RPMI 1640 containing 10% fetal calf serum, 2 mM-glutamine, 5 × 10^-5 M-β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. After this time effector cells were harvested, counted and used in chromium release assays.

Target cells and cytotoxicity assays. Target cells for cytotoxicity assays were BCH4 cells, a line of BALB/c embryo fibroblasts persistently infected with the Long strain of RS virus (Fernie et al., 1981) or BALB/c fibroblasts (Taylor et al., 1985) infected in suspension with 2.5 p.f.u./cell of the A2 strain of RS virus for 90 min at 37 °C and incubated for a further 21 h to allow viral protein expression before use in CTL assays. The mouse fibroblast line L-929 (H-2^k) was infected as described above. YAC-1 cells were included to detect natural killer (NK) activity and uninfected BALB/c fibroblasts were used as a control. Suspensions of infected and control target cells were prepared by trypsinization of monolayers. Cells were labelled as a pellet with 100 μCi Na_2^{63}CrO_4 (Dupont) per 10^6 cells for 90 min at 37 °C, and washed three times in medium before 5 × 10^3 targets were plated out into each well of a V-bottomed 96-well plate. Effector cells were added to give a final volume of 150 μl per well; microtitre plates were
centrifuged and incubated for 4 h at 37 °C in 5% CO₂/air. The percentage specific release of ⁶⁷Cr = ([c.p.m. experimental − c.p.m. spontaneous]/(c.p.m. maximum − c.p.m. spontaneous)) × 100. Spontaneous release was the value obtained with medium alone and maximum release was determined following incubation of target cells with 1% SDS. Each value was the mean of three replicates.

Depletion of CD8⁺ cells. Washed effector cells were incubated in Lyt 2⁺-specific MAb (clone YTS 169.4, Seralab) diluted 1:125 in 2% FCS in RPMI for 1 h at 4 °C (100 µl per 10⁶ cells). The cells were washed twice in PBS and incubated with rabbit complement (Sigma) diluted 1:15 in 2% FCS in RPMI (100 µl per 10⁶ cells) for 1 h. The cells were washed as before and used directly in a chromium release assay.

**Results**

**Antibody response in mice vaccinated with vv expressing mutant F protein**

To determine whether the mutations within the VA-FT, VA-FR47 and VA-FS1 to VA-FS6 recombinants (shown in Table 1) influenced the immunogenic properties of the F protein, the antibody responses induced by the constructs were examined in mice. The vv recombinants which included a replacement of a Phe to a Ser at amino acid 237, either alone or in combination with other mutations, inhibited the transport of the F protein to the cell surface before F protein reached the medial Golgi compartment (López et al., 1996). Sera were tested by ELISA for the presence of antibodies to vv and to purified RS virus F protein (Table 1). All vaccinated mice developed antibodies to vv. However, only the mice vaccinated with VA-F, VA-FT, VA-FS1 and VA-FS4 recombinants, which resulted in cell surface expression of the F protein, developed antibody which recognized purified F protein. The amino acid change (Phe to Ser) at position 237 was common to all of the recombinants which failed to induce an antibody response to the F protein in mice. Sera from mice given VA-F and VA-FT neutralized virus infectivity, whereas sera from mice given VA-FR47, a recombinant containing a mutation which causes the F protein to be retained within the cell, did not contain neutralizing antibody (Table 1).

Further characterization of sera from vaccinated animals by Western blotting showed that VA-F, VA-FT and VA-FR47 were all able to elicit antibodies that recognized the denatured F₁ subunit of either Long or R47F/4 viruses (Table 1). In addition, sera from mice inoculated with recombinants VA-FS1 to VA-FS6 all contained antibodies that reacted by Western blot with the F₁ subunit.

**Generation of a CTL response**

It has been shown previously that recombinant vv expressing the F protein of RS virus primes BALB/c mice for a RS virus specific CTL response (Pemberton et al., 1987; Alwan & Openshaw, 1993). Therefore, the ability of vv recombinants expressing mutant forms of the F protein to induce CTL was investigated. Secondary CTLs were generated following in vitro stimulation of splenocytes from mice infected with either RS virus or the vv recombinants. Lymphocytes from mice inoculated with RS virus, VA-F, VA-FT or VA-FR47 specifically lysed BCH4 cells but not uninfected BALB/c fibroblasts (Fig. 1). There was less than 6% lysis of the NK sensitive YAC-1 cell line (data not shown), suggesting that the lysis observed was not due to NK cells. Similarly, no more than 5% lysis was observed for either uninfected cells or RS virus-infected MHC mismatched L-929 (H-2d) cells. Pre-incubation of the effector cells with Lyt 2⁺ (CD8-specific) MAb and complement eliminated or greatly reduced the cytotoxic activity of the lymphocytes (specific lysis less than 2%), demonstrating that the CTL response induced by the recombinant vv was mediated by CD8⁺ cells (Table 2).

Further experiments demonstrated that splenocytes from mice vaccinated with VA-FS1, VA-FS2, VA-FS3, VA-FS4, VA-FS5 or VA-FS6 were all able to generate RS virus-specific CTL responses (Fig. 2). Lysis of RS virus-infected BALB/c fibroblasts and of BCH4 (H-2k) cells was considerably greater than that of the uninfected BALB/c fibroblasts or of virus-infected L-929 (H-2k) cells, indicating that lysis was mainly MHC-restricted. However, where appreciable lysis of RS virus-infected L-929 MHC-mismatched targets was observed, lysis of YAC-1 cells was also seen indicating that in some of the experiments shown in Fig. 2 up to 35% of the total lytic activity may have been mediated by NK cells. As seen previously, lysis of infected cells was either abolished or

<table>
<thead>
<tr>
<th>Virus that mice were inoculated with:</th>
<th>Specific lysis (%) by lymphocytes* treated with:</th>
<th>Complement alone</th>
<th>Complement + Lyt 2⁺ antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VA-F</td>
<td>22</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VA-FT</td>
<td>22</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VA-FR47</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>VA-FS1</td>
<td>43</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>VA-FS2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>VA-FS3</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VA-FS4</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VA-FS5</td>
<td>40</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>VA-FS6</td>
<td>38</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* Spleen cells obtained from mice inoculated i.n. with RS virus or i.p. with recombinant vv 4 weeks previously.
ND, Not determined.
Immune responses to RS virus F protein mutants

greatly reduced if the effector cells were incubated first with Lyt 2+ MAb and complement, indicating that the predominant effector cells were CD8+ T cells (Table 2).

Lysis of RS virus-infected cells by effector cells treated with antibody to Lyt 2+ and complement may have been mediated by CD4+ CTL or possibly residual CD8+ CTL.

Fig. 2. Cytotoxic activity of splenocytes from mice vaccinated with VA-F, VA-FS1, VA-FS2, VA-FS3, VA-FS4, VA-FS5 or VA-FS6, tested at decreasing effector:target (E:T) ratios on uninfected BALB/c fibroblasts (○), RS virus-infected BALB/c fibroblasts (●), BCH4 cells (□) or RS virus-infected L929 cells (●).
Table 3. Ability of vv recombinants to protect mice against RS virus infection 4 and 16 weeks after vaccination

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Vaccine</th>
<th>Antibody response to RS virus*</th>
<th>RS virus titre in lungs (log_{10} p.f.u./g)†</th>
<th>Lung wash cell count (log_{10} cells/ml)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VA-F</td>
<td>44 ± 0.4 52 ± 0.5</td>
<td>&lt; 1.7‡ (0/5)§</td>
<td>54.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>VA-FT</td>
<td>38 ± 0.8 47 ± 0.2</td>
<td>&lt; 1.7‡ (0/5)</td>
<td>54.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>VA-FR47</td>
<td>&lt; 1.5 24 ± 0.7</td>
<td>40 ± 0.3 (5/5)</td>
<td>33.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>&lt; 1.5 &lt; 1.5</td>
<td>46 ± 0.1 (5/5)</td>
<td>43 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>VA-F</td>
<td>4.5 ± 0.5 ND</td>
<td>&lt; 1.7‡ (1/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS1</td>
<td>3.7 ± 0.4 ND</td>
<td>40 ± 0.3 (5/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS2</td>
<td>&lt; 1.5 ND</td>
<td>22 ± 0.8‡ (2/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS3</td>
<td>1.7 ± 0.5 ND</td>
<td>50 ± 0.4 (5/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS4</td>
<td>4.1 ± 0.3 ND</td>
<td>44 ± 0.2 (5/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS5</td>
<td>&lt; 1.5 ND</td>
<td>43 ± 0.3 (5/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VA-FS6</td>
<td>&lt; 1.5 ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean ± SD log_{10} titre of antibody to RS virus determined by ELISA at 4 weeks (Expt no. 1) or 16 weeks (Expt no. 2) after vaccination, and 7 days after RS virus challenge.
† Mean ± SD titre of RS virus in lungs 5 days after challenge.
‡ Probability of difference in virus titres between vaccinated and control mice < 0.0001, calculated by Student's t-test.
§ No. of mice infected/total.
|| Lung wash cell counts 7 days after RS virus challenge.
¶ Lung wash cell counts in vaccinated mice were significantly different by Student's t-test from those of unvaccinated controls, P < 0.02.
ND, Not determined.

However, since lysis of YAC-1 cells was observed, it is likely that the remaining activity was due to NK cells. There was no significant difference between lysis of RS virus-infected cells by untreated lymphocytes and those treated with or without complement alone.

**Ability of vv recombinants to protect against RS virus infection at different times after vaccination**

Protection against RS virus infection was examined following vaccination with vv recombinants expressing intact or mutant forms of the F protein. Four weeks after vaccination with VA-F, VA-FT or VA-FR47, mice vaccinated with recombinant VA-FR47 failed to develop antibodies to RS virus which could be detected by ELISA (Table 3). Although VA-FR47 was unable to induce antibodies detectable by ELISA, there was evidence that priming had occurred as the antibody titre increased following RS virus challenge when compared with unvaccinated controls. However, this priming effect of the humoral immune response was insufficient to confer protection and the titres of the virus recovered from the lungs of these mice were not significantly different from unvaccinated controls (Table 3).

In a separate experiment, the VA-FS1 to VA-FS6 mutants were also examined for their ability to protect mice 16 weeks after vaccination. Mice vaccinated with VA-FS2, VA-FS3, VA-FS5 and VA-FS6 did not develop an RS virus antibody response that was detectable by ELISA. Furthermore, these animals were not protected against RS virus infection (Table 3). Mice given VA-FS1 and VA-FS4, which had either or both of the 223 (Phe to Leu) or 262 (Asn to Tyr) mutations, were able to generate RS virus antibodies and were protected against RS virus infection as shown by the reduction in RS virus titre in the lungs when compared with unvaccinated controls (Table 3).

The effect of vaccination on the development of pneumonic lesions was studied by counting the number of cells in lung washes obtained 7 days after RS virus challenge and by microscopic examination of lung sections. There was a three- to fourfold increase in the number of cells in the lung washings obtained from all vaccinated mice, 7 days after RS virus infection, compared with unvaccinated, RS virus-infected animals (Table 3). Lung lesions from mice vaccinated 4 weeks previously were examined 7 days after RS virus challenge and comprised peribronchiolar and perivascular accumulations of lymphocytes (Fig. 3C, D). Virus challenge of mice which had been vaccinated with a recombinant control (VA-5C) resulted in only a small amount of cellular infiltrate (Fig. 3B), similar to that seen in unvaccinated, RS virus-infected mice (unpublished observations), whereas there was no cellular infiltrate in lung sections from mice inoculated with virus-free cell supernatant (Fig. 3A). The cellular infiltrate was much greater in mice that had been vaccinated with the VA-F or VA-FR47 recombinant prior to challenge (Fig. 3C, D respectively) when compared with RS virus-infected controls.
Table 4. Ability of recombinant vv to protect mice against RS virus infection 6 and 10 days after vaccination

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Vaccine</th>
<th>Route</th>
<th>Day of challenge</th>
<th>RS virus titre in lungs* (log_{10} p.f.u./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VA-FT</td>
<td>i.n.</td>
<td>6</td>
<td>1.9 ± 0.6†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>1.9 ± 0.6†</td>
</tr>
<tr>
<td></td>
<td>VA-FS2</td>
<td>i.n.</td>
<td>6</td>
<td>2.0 ± 0.9†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>2.2 ± 1.2†</td>
</tr>
<tr>
<td></td>
<td>VA-β Gal</td>
<td>i.n.</td>
<td>6</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>VA-F</td>
<td>i.n.</td>
<td>10</td>
<td>&lt; 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>10</td>
<td>3.0 ± 0.5§</td>
</tr>
</tbody>
</table>

* Mean ± SD titre of RS virus in lungs 5 days after challenge.
† P < 0.005 by Student’s t-test.
‡ P < 0.05 by Student’s t-test.
§ P < 0.0005 by Student’s t-test.

Resistance to RS virus infection in mice primed with recombinant vv expressing the M2 (22K) protein, which is the major CTL antigen in BALB/c mice (Openshaw et al., 1990), was short-lived and mediated by primary CTLs, i.e. those induced by vaccination and not requiring restimulation by RS virus (Connors et al., 1991). Thus, protection against RS virus infection observed in the lungs of mice vaccinated 10 days previously had largely waned by day 28 post-vaccination. Since recombinant vv expressing mutant forms of the F protein that remained intracellular primed CTL but did not protect mice challenged at 28 days post-vaccination or later, the ability of these recombinants to protect against infection when mice were challenged 6–10 days after vaccination was studied. Mice were, therefore, vaccinated with VA-F and VA-FT expressing native F protein or VA-FS2, which contained only the mutation responsible for the retention of the F protein within the cytoplasm. A significant reduction in lung virus titre was observed following RS virus challenge in mice vaccinated either i.n. or i.p. with VA-F, VA-FT or VA-FS2 6 or 10 days previously when compared with the control animals vaccinated with recombinant vv expressing the β-galactosidase protein (Table 4).

Discussion

Vaccinia virus recombinants expressing mutant forms of the F protein of RS virus in which both the proteolytic processing of the F_0 precursor and its transport to the
cell surface were inhibited did not induce a neutralizing antibody response but did prime CTLs. These observations allowed the relative contribution of these components of the immune response in resistance against RS virus infection to be studied. Priming of CD8+ CTL, in the absence of a neutralizing antibody response, induced only a transient resistance to RS virus infection in BALB/c mice and suggests that prolonged resistance to infection requires the production of a protective antibody response. Although vv recombinants expressing F genes containing Phe\textsuperscript{237} → Ser induced antibodies that reacted with denatured F protein in a Western blot, only those mutants able to induce neutralizing antibodies or antibodies which recognized native F protein in ELISA protected against RS virus infection. This indicates that cleavage of F\textsubscript{0} and correct folding of the F protein are essential for the induction of a protective antibody response.

The transient resistance to infection seen in vaccinated mice was similar to that observed by Connors et al. (1991) who showed that resistance to RS virus infection induced by recombinant vv expressing the M2 and N proteins of RS virus is relatively short-lived. Thus, mice vaccinated simultaneously by the i.n. and i.p. routes with these recombinants exhibited significant resistance to RS virus infection after 9 days, but this resistance, which was mediated by a primary CTL response (Connors et al., 1992), had largely waned 28 days after vaccination.

Vaccinia virus recombinants expressing viral proteins have been shown to induce CTL responses, which vary in their ability to protect against infection. Complete long-lasting protection against murine cytomegalovirus can be achieved by vaccinating mice with a vv recombinant expressing the immediate-early protein pp89 (Del Val et al., 1991). In contrast, although CTLs were induced following vaccination of mice with a vv recombinant expressing a minigene of the influenza nucleoprotein, the CTLs did not accelerate influenza virus clearance and did not protect (Lawson et al., 1994). The nature of the expressed protein may dictate whether a protective response evolves, though the reasons for this are unclear. Perhaps a protein containing a greater number of CTL epitopes induces a more protective immune response because more CTL cells are activated. Alternatively, the site of virus replication or the target host cell may influence the capacity of CTLs to mediate protection. Furthermore, use of vv vectors to introduce RS virus proteins to the immune system may affect the CTL response. It is possible that the presence of many vv peptides capable of binding to the MHC proteins could alter the immunodominance of peptides normally presented to CTL when only RS virus proteins are available, and thus modify precursor frequency and the effectiveness of a memory CTL response.

The MHC background of the host animal also has a direct effect on the CTL response as the amino acids within the peptide binding groove of each MHC molecule will determine which viral peptides can be accommodated and thus presented to CTL (for review see Rammensee et al., 1995). Protection against lymphocytic choriomeningitis virus (LCMV) is virtually exclusively mediated by virus-specific CTLs (Zinkernagel & Welsh, 1976), and Hany et al. (1989) demonstrated that different levels of CTL activity and in vivo protection could be generated depending on the MHC–protein combination. Splenocytes from mice of different MHC backgrounds which had been primed with LCMV in vivo were stimulated in vitro with vv recombinants expressing the N or G protein of LCMV. A protein–MHC combination which resulted in high levels of specific lysis was generally associated with longer-lasting protection than protein–MHC combinations which resulted in only low CTL responses.

The transient nature of the resistance to RS virus induced by VA-FS2 is consistent with the time-course of a primary CTL response (Bennink & Yewdell, 1990). These studies suggest that CTLs are more effective at restricting RS virus replication, at least within the first 5 days of infection, than CTLs generated from a memory population. Memory T cells are not immediately available to deal with a secondary infection and it takes 3 to 5 days for memory CTL precursors to differentiate to a stage where they can mediate effector functions (Doherty & Sarawar, 1994). Therefore, restimulation of memory CTL, whilst not affecting peak titres of virus, may accelerate virus clearance from immunized mice. This response may be of particular importance in children where virus shedding may occur for up to 3 weeks (Hall et al., 1976). It remains to be determined whether accelerated clearance of RS virus from the lungs of vaccinated mice, when compared with controls, can be observed at times later than 5 days post-infection. Studies on the adoptive transfer of RS virus-primed memory CTLs have shown that virus may be cleared from the lungs of persistently infected X-irradiated mice, provided that the CTLs were administered early after infection (Cannon et al., 1987). Cells given 2 weeks after virus infection were unable to restrict virus replication. A later study, involving transfer of primed T cells to immunocompetent BALB/c mice soon after virus infection, also showed that virus could be cleared by CD8+ cells but that T cell transfer was associated with enhanced lung pathology, respiratory distress and high mortality (Cannon et al., 1988). Perhaps stimulating memory CTLs in vitro selectively amplifies a CTL population which has stronger antiviral activities or is more highly activated than CTLs induced by a single immunization in vivo. Alternatively, it is possible that the number of
adoptively transferred RS virus-specific CTLs exceeded that induced after RS virus challenge of vaccinated mice. Nevertheless, there was a marked influx of lymphocytes into the lungs of mice vaccinated with vv recombinants expressing the RS virus F protein in this study and the lymphocyte infiltration was greater than that observed in the lungs of control mice. Following RS virus challenge, the number of lymphocytes was 25-fold greater in bronchoalveolar lavage from mice primed with vv expressing the F protein compared with mice given wild-type vv (Openshaw et al., 1992). In these studies, CD8+ T cells outnumbered CD4+ T cells by 4:3. The rate of loss of CD45RB from the CD8+ T cells was maximal 9 days after RS virus challenge, indicating that the cells were activated.

In conclusion, vaccines against RS virus that rely solely on an MHC class I restricted CTL response may provide only transient resistance to infection, although they may reduce the severity of illness by enhancing the clearance of virus later in infection. It is likely that to achieve long-term protection against RS virus infection, vaccines will need to induce a protective humoral response.

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### References


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