Construction and characterization of recombinant vaccinia viruses co-expressing a respiratory syncytial virus protein and a cytokine

Teresa R. Johnson,1 Julie E. Fischer1 and Barney S. Graham1, 2

Departments of Microbiology and Immunology1 and Medicine2, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Recombinant vaccinia viruses are well-characterized tools that can be used to define novel approaches to vaccine formulation and delivery. While vector co-expression of immune mediators has enormous potential for optimizing the composition of vaccine-induced immune responses, the impact on antigen expression and vector antigenicity must also be considered. Co-expression of IL-4 increased vaccinia virus vector titres, while IFN-γ co-expression reduced vaccinia virus replication in BALB/c mice and in C57BL/6 mice infected with some recombinant viruses. Protection against respiratory syncytial virus (RSV) challenge was similar in mice immunized with vaccinia virus expressing RSV G glycoprotein and IFN-γ, even though the replication efficiency of the vector was diminished. These data demonstrate the ability of vector-expressed cytokine to influence the virulence of the vector and to direct the development of selected immune responses. This suggests that the co-expression of cytokines and other immunomodulators has the potential to improve the safety of vaccine vectors while improving the immunogenicity of vaccine antigens.

Introduction

Poxvirus genomes have been well characterized (Joklik, 1985; Mars & Beaud, 1987), facilitating the construction of recombinant viruses with the capacity to express multiple genes (Mackett et al., 1982). Recombinant poxvirus vectors are important tools for vaccine development and the study of microbial pathogenesis (Coupar et al., 1987; Ramshaw et al., 1987; Perkus et al., 1995). The development of recombinant virus vectors that co-express a viral antigen and an immune modulator may serve to amplify protective immune effectors or to down-regulate immune responses that correlate with adverse disease profiles. This approach to immunomodulation during immunization has been applied to a variety of pathogens in both human and animal studies (Davis et al., 1996; Hsu et al., 1994; Mathews et al., 1994; Andrew et al., 1986; Doherty et al., 1989; Sharma et al., 1996; Takao et al., 1997; An & Whitten, 1997; Bembridge et al., 1998b; Aung et al., 1999), demonstrating that the generation of protective antigen-specific humoral and cell-mediated immune responses can be influenced at the time of vaccination.

The immunology of poxvirus infection is complex. Vaccinia virus is a potent inducer of IFN production, nitric oxide synthase (iNOS) and other antiviral immune mediators (Harris et al., 1995; Karupiah et al., 1993; Ruby & Ramshaw, 1991). In addition, poxviruses have developed multiple mechanisms of immune evasion such as the incorporation of host immune response genes into the viral genome. In vitro infection of human cell lines and chicken chorioallantoic membrane (CAM) cultures has revealed that secreted forms of several cytokine receptors are encoded by many poxviruses and include the type II tumour necrosis factor receptor (Hu et al., 1994), IFN-γ receptors (Alcamí & Smith, 1995) and the crmA protein (Palumbo et al., 1994). Viral expression of these proteins may potentially alter the proposed effects of cytokines expressed by recombinant vectors. Studies in CAM cultures (Palumbo et al., 1994) and in established mouse models (Boyle & Coupar, 1986; Lee et al., 1992; Karupiah et al., 1991; Palumbo et al., 1994) have shown that the insertion of foreign genes into the vaccinia virus genome modifies the growth kinetics and dissemination patterns of the vector, which, in turn, may alter the production of foreign genes. Thus, the effects of genetic manipulations on immunogenicity and growth of recombinant...
virus vectors must be examined carefully before conclusions can be made about the ability of the expressed immune mediator to modulate responses specific for the target antigen.

Respiratory syncytial virus (RSV) disease in mice is primarily immune-mediated, with little direct cytopathicity resulting from virus replication (Graham et al., 1991; Graham, 1995; Tang & Graham, 1997; Piedra et al., 1989). The immunization of mice with the RSV G glycoprotein results in the generation of type 2 CD4+ T cells (Alvan & Openshaw, 1993). G-primed mice exhibit illness and pulmonary eosinophilia following RSV challenge (Hancock et al., 1996; Openshaw et al., 1992; Srikitkhachorn & Braciale, 1997a), with the secreted form of G predisposing for more severe disease (Johnson et al., 1998; Bembridge et al., 1998a). Depletion of IFN-γ-producing CD8+ T cells or neutralization of IFN-γ amplifies the type 2-oriented immune responses (Srikitkhachorn & Braciale, 1997a; Hussell et al., 1997). Therefore, interference with cytokine function during induction of these RSV G-specific immune responses may modulate the disease profile following challenge.

In order to test the ability of specific cytokines to influence immune responses against individual RSV proteins, a panel of recombinant vaccinia viruses co-expressing an RSV protein and a murine cytokine was constructed. The constructs were then characterized in vivo for the ability to replicate and disseminate in mice and compared with non-cytokine-expressing vectors to determine whether cytokine expression affected virus growth patterns. Finally, the ability of IFN-γ to modulate induction of type 2 immune responses during immunization with secreted G was examined by RSV challenge of mice immunized with an IFN-γ-co-expressing vaccinia virus.

Methods

Cell, virus and plasmid stocks. BSC40 cells were obtained from Shiu-Lok Hu (University of Washington, Seattle, WA, USA) and maintained in Eagle’s modified medium supplemented with 10% foetal calf serum, glutamine and antibiotics (10% EMEM). 143B, a thymidine kinase-deficient osteosarcoma cell line, was obtained from the ATCC and maintained in 10% EMEM containing 50 μg/ml bromodeoxyuridine (Sigma). HEP-2 cells were purchased from the ATCC and maintained in 10% EMEM. Recombinant vaccinia virus expressing RSV F (vacF) was obtained from Peter Collins (NIH, Bethesda, MD, USA). Constructs expressing full-length RSV G (vvGwt) and secreted G (vvGs) were gifts of Gail Wertz (University of Alabama at Birmingham, Birmingham, AL, USA). A control virus expressing β-galactosidase (vac-lac) was obtained from Bernard Moss (NIH, Bethesda, MD, USA). The foreign genes of these recombinant viruses were inserted at the HindIII site of the vaccinia virus genome, interrupting the virus thymidine kinase (TK) gene. A stock of human RSV strain A2 was grown in HEP-2 cells as described previously (Graham et al., 1988). All cell and virus stocks were determined to be free of mycoplasma contamination by PCR (ATCC).

Plasmids pFBX-mIL4 and pFBX-mIFNγ, expressing murine IL-4 and IFN-γ, respectively, were gifts of Ian Ramshaw (John Curtin School of Medical Research, Canberra, Australia). The plasmids were constructed in such a way that the cytokine gene was flanked by vaccinia virus HindIII-F sequences (Andrew et al., 1989). The insert contained the herpes simplex virus TK gene, allowing selection of TK+ recombinant viruses (Andrew et al., 1989). Stocks of each plasmid were established by transfection of E. coli DH5α and isolation of the plasmid DNA using Plasmid Maxi-Prep kits according to the kit protocol (Qiagen).

Generation of cytokine-co-expressing viruses. Recombinant vaccinia viruses expressing both an RSV protein and a murine cytokine were constructed according to the published protocol of Coupar et al. (1988) with modifications. Subconfluent monolayers of 143B cells were grown in 10% EMEM. The medium was removed and the cells were incubated in EMEM containing glutamine and antibiotics but no foetal calf serum (0% EMEM) for 1 h. After removing the medium, 5 × 106 p.f.u. vaccinia virus (m.o.i. = 0·1) was adsorbed to the cells for 3 h at room temperature. Plasmid DNA was precipitated for 45 min and, without removing the virus-containing medium, was added to vaccinia virus-infected 143B cells and incubated at room temperature for 45 min. 0% EMEM was then added to each well and the plates were incubated at room temperature for an additional 2·5 h. All medium was then removed from the wells and selection medium was added. In order to select TK+ cytokine-expressing recombinant viruses, 10% EMEM containing 3 μM methotrexate, 15 μM thymidine, 50 μM adenosine, 50 μM guanosine and 10 μM glycine (10% EMEM–MTAGG) was used. Supernatants from the infected cultures were serially passaged to fresh 143B cells every second day for 8 days and grown in 10% EMEM–MTAGG.

Plaque purification of the recombinant virus began 10 days post-infection as follows. On day 10, 143B cells were infected with day 8 culture supernatant. The cells were overlaid with 10% EMEM–MTAGG containing 0·5% agarose. Well-isolated plaques were selected on day 12, and the culture material was resuspended in 10% EMEM. To ascertain that the isolated virus expressed the cytokine and was not contaminated with non-cytokine-expressing virus, fresh 143B cells were infected with supernatant from the resuspended plaque and grown in 10% EMEM without selection. After 2 days of growth, the supernatant was tested by ELISA for cytokine production by using commercially available kits (Endogen). Cytokine-expressing plaques were then infected onto fresh 143B cells and overlaid with 10% EMEM–MTAGG–agarose. After four rounds of plaque purification under selection and testing at each round, positive plaques were considered to be clones.

Growth and purification of recombinant vaccinia viruses. Virus stocks were generated by infecting 143B cells with day 26 supernatant (from ELISA screening culture after fourth round of plaque purification). The stocks were grown under 10% EMEM–MTAGG selection for 3–4 days. The culture medium was harvested and frozen at −70 °C until purification. The vaccinia virus was purified by density gradient centrifugation on potassium tartrate gradients as described previously (Johnson et al., 1998). The level of cytokine production by each purified vaccinia virus stock was quantified by cytokine-specific ELISA using supernatants from BSC40 cells 2 days post-infection.

In vivo replication and dissemination kinetics of cytokine-expressing vaccinia viruses. Pathogen-free 0- to 8-week-old BALB/c and C57BL/6 mice were obtained from Charles Rivers Laboratories and housed in a barrier facility in accordance with the Guide for the Care and Use of Laboratory Animals. IL-4-deficient mice (IL-4−/−) were generated by targeted disruption of the IL-4 gene as described previously (Kopf et al., 1993) on a C57BL/6 genetic background and were a gift of O. Kanagawa (Washington University, St Louis, MO, USA).

Mice were infected with 5 × 105 p.f.u. (in 0·05 ml) recombinant vaccinia virus by intradermal inoculation at the base of the tail and were monitored daily for lesion formation. In order to establish the kinetic patterns of vaccinia virus replication and dissemination, mice were
sacrificed by CO₂ narcosis and cervical dislocation at the indicated time-points after infection. Tissues were removed, placed in 10% EMEM and frozen at −70 °C. Virus titres in each tissue sample were measured by plaque assay on BSC40 monolayers. Briefly, the samples were quick-thawed and ground with a mortar and pestle. Serial 10-fold dilutions of clarified supernatants were used to infect the monolayers in triplicate and cultures were grown under 0.75% methylcellulose in 10% EMEM. Cells were formalin-fixed 2 days after infection and stained with 2% crystal violet/40% methanol and plaques were counted under a dissecting microscope. Data are represented as the geometric mean log10 p.f.u. per gram of tissue at the dilution that produced more than five plaques per well.

Mouse immunization and challenge. BALB/c mice were immunized intradermally at the base of the tail with 5 × 10⁸ p.f.u. vaccinia virus. Six weeks after immunization (4 weeks after clearance of cutaneous lesions), the mice were anaesthetized and infected intranasally with virus. Six weeks after immunization (4 weeks after clearance of cutaneous lesions), the mice were anaesthetized and infected intranasally with virus. Balb/c mice were infected with 1 ml containing 10⁷ p.f.u. live RSV. Mice were weighed and illness was scored daily as described previously (Johnson et al., 1998).

Immune responses following RSV challenge. The abilities of vvGs and vvGs−IFN-γ immunization to protect against virus replication following RSV challenge were compared at days 4 and 7 post-challenge as described previously (Johnson et al., 1998). Levels of IL-4, IL-5, IL-13, IFN-γ and eotaxin protein in day 4 lung supernatants were measured using commercially available ELISA kits (R&D Systems). Mice were sacrificed 7 days after challenge (around the peak of lymphocyte recruitment to the lung). Bronchoalveolar lavage (BAL) and differential cell staining and counts were performed as described previously (Johnson et al., 1998).

Analysis of RSV- and vaccinia virus-specific antibody responses. Serum was collected by retroorbital puncture 1 day prior to RSV challenge and 6 weeks after challenge. Titres and isotype profiles of G-specific antibodies were determined as described previously (Johnson et al., 1998). Titres of neutralizing antibodies to vaccinia virus in pre-challenge sera were measured by plaque-reduction neutralization assays, as described previously (Graham et al., 1988).

Table 1. Panel of RSV–cytokine recombinant vaccinia viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>HindIII-J insert</th>
<th>HindIII-F insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>vac-lac</td>
<td>β-Galactosidase</td>
<td>None</td>
</tr>
<tr>
<td>vac-lac–IL4</td>
<td>β-Galactosidase</td>
<td>Murine IL-4</td>
</tr>
<tr>
<td>vac-lac–IFN-γ</td>
<td>β-Galactosidase</td>
<td>Murine IFN-γ</td>
</tr>
<tr>
<td>vacF</td>
<td>RSV F</td>
<td>None</td>
</tr>
<tr>
<td>vacF–IL4</td>
<td>RSV F</td>
<td>Murine IL-4</td>
</tr>
<tr>
<td>vacF–IFN-γ</td>
<td>RSV F</td>
<td>Murine IFN-γ</td>
</tr>
<tr>
<td>vvGwt</td>
<td>RSV G</td>
<td>None</td>
</tr>
<tr>
<td>vvGwt–IL4</td>
<td>RSV G</td>
<td>Murine IL-4</td>
</tr>
<tr>
<td>vvGwt–IFN-γ</td>
<td>RSV G</td>
<td>Murine IFN-γ</td>
</tr>
<tr>
<td>vvGs</td>
<td>Secreted G</td>
<td>None</td>
</tr>
<tr>
<td>vvGs–IFN-γ</td>
<td>Secreted G</td>
<td>Murine IFN-γ</td>
</tr>
</tbody>
</table>

Fig. 1. Vaccinia virus takes more than 2 weeks to be cleared from the site of injection, but has limited dissemination. BALB/c mice were infected with vaccinia virus intradermally at the base of the tail. At the indicated times post-infection, mice were sacrificed and tissues were harvested. Vaccinia virus titres were measured by plaque assay on BSC40 monolayers. The data are represented as means ± SEM log₁₀(p.f.u./g tissue) for 12 mice from three separate experiments.
Mice were infected with recombinant vaccinia viruses and virus titres were examined at day 7 post-infection by plaque assay. The data are represented as the mean ± SEM \( \log_{10} \) p.f.u./g tissue, with the probability \( P \) compared to non-cytokine-secreting vector given in parentheses, for nine to twelve mice pooled from three separate experiments. The limit of detection was 1-5 \( \log_{10} \) p.f.u./g tissue. Numbers of mice showing detectable vaccinia virus are given in square brackets.

<table>
<thead>
<tr>
<th>Virus</th>
<th>BALB/c Tail</th>
<th>Spleen</th>
<th>Ovary</th>
<th>C57BL/6 Tail</th>
<th>Spleen</th>
<th>Ovary</th>
<th>IL-4(^{-/-}) Tail</th>
<th>Spleen</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>vac-lac</td>
<td>5.1 ± 0.4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.9 ± 0.6</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>5.8 ± 0.2</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vac-lac-IL4</td>
<td>5.7 ± 0.1</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>5.8 ± 0.6</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>6.4 ± 0.1</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vac-lac-IFN-γ</td>
<td>2.7 ± 0.4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>5.8 ± 0.0</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>5.3 ± 0.4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vacF</td>
<td>5.7 ± 0.4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>6.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>[2/9]</td>
<td>5.6 ± 0.3</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vacF-IL4</td>
<td>4.0 ± 0.3</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>6.1 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>[0/18]</td>
<td>5.8 ± 0.2</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vacF-IFN-γ</td>
<td>1.9 ± 0.3</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.7 ± 0.4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>2.3 ± 0.2</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vvGwt</td>
<td>6.2 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>&lt; 1.5</td>
<td>5.9 ± 0.1</td>
<td>1.9 ± 0.01</td>
<td>[2/9]</td>
<td>5.9 ± 0.2</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vvGwt-IL4</td>
<td>7.0 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>&lt; 1.5</td>
<td>7.5 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>[4/9]</td>
<td>5.8 ± 0.5</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>vvGwt-IFN-γ</td>
<td>4.5 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td>&lt; 1.5</td>
<td>6.1 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>[2/9]</td>
<td>6.0 ± 0.1</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
</tr>
</tbody>
</table>

Vaccinia viruses produced 13,600 ± 1200 pg/ml IL-4 and the IFN-γ-expressing viruses produced 66,200 ± 2100 pg/ml IFN-γ.

**Vaccinia virus infection of BALB/c mice results in minimal dissemination and requires more than 2 weeks to clear from the site of injection**

BALB/c mice were infected with vac-lac, vacF or vvGwt vaccinia viruses expressing no cytokine. Mice were euthanized at days 4, 7, 10 and 14 after infection and tails, lungs, brain, liver, spleen, inguinal lymph nodes, heart, thymus and ovaries were removed. Vaccinia virus titres were examined in each sample. When dissemination was examined, little virus was detected in tissues other than the tail (Fig. 1). Titres were similar in vac-lac-, vacF- and vvGwt-infected mice. Thus, expression of the RSV F or G protein did not alter significantly the magnitude, tropism or kinetics of vaccinia virus growth in mice. Notably, 2 weeks post-infection, vaccinia virus was still detectable at the site of injection. This becomes important when these viruses, potent inducers of both innate and adaptive immune responses, are used as experimental vaccine vectors. For example, RSV challenge of vaccinia virus-immunized mice prior to resolution of innate immune responses and primary vaccinia virus-specific responses may complicate the interpretation of subsequent RSV-specific immune responses.

**Co-expression of IFN-γ decreases virus titres, while IL-4 co-expression with RSV G increases virus titres and dissemination**

BALB/c mice were infected with vac-lac, vacF and vvGwt vaccinia virus vectors expressing no cytokine or co-expressing IL-4 or IFN-γ and virus dissemination and clearance were examined (Table 2). Relative to non-cytokine-producing control viruses, co-expression of IFN-γ in vac-lac, vacF and vvGwt vectors decreased virus titres in the tail significantly (Table 2; \( P \leq 0.02 \)). Since dissemination of non-cytokine-expressing vectors was minimal (Fig. 1), the impact of IFN-γ co-expression on dissemination could not be evaluated. Conversely, co-expression of IL-4 in vac-lac or vvGwt vectors increased virus titres in the tail significantly (Table 2). Co-expression of IL-4 by the vvGwt vaccinia virus increased dissemination, resulting in detectable virus in the spleen and ovaries, with virus titres significantly greater than those of vvGwt-infected mice (Table 2). Thus, expression of either IL-4 or IFN-γ may alter the magnitude and site of vaccinia virus replication, either directly or indirectly. However, the impact
of the cytokine on vector replication may be affected by the expression of a recombinant antigen, as demonstrated by the ability of IL-4 to increase vaccinia virus titres and dissemination when co-expressed with RSV G, but not with RSV F (Table 2).

Growth patterns of some antigen–cytokine recombinant vaccinia viruses can differ between BALB/c, C57BL/6 and IL-4-deficient mice

In order to determine whether genetic differences between different strains of mice affect replication of vaccinia virus, BALB/c and C57BL/6 mice were infected with vac-lac, vacF, vvGwt and the IL-4- and IFN-γ-co-expressing recombinant viruses. The ability of co-expressed cytokine to compensate for global deficiency was examined concomitantly by the infection of IL-4−/− mice. Minimal dissemination was observed in all three strains of mice, with low levels of virus detected in the spleen and ovaries (Table 2). Differences in vac-lac and vacF titres were not significant between the strains. Titres of vac-lac–IFN-γ, vacF–IL-4 and vvGwt–IFN-γ in the tail were significantly lower in BALB/c mice relative to both C57BL/6 and IL-4−/− mice (Table 2; P < 0.02). BALB/c mice also had significantly lower vacF–IFN-γ titres than did C57BL/6 mice (P < 0.01). Though not statistically significant, vac-lac titres in C57BL/6 mice were lower than titres in BALB/c mice. This may reflect differences in NK cell activity and IFN-γ production between mouse strains and may mask the effects of IFN-γ co-expression in C57BL/6 mice. When virus titres in IL-4−/− mice and C57BL/6 controls were compared, significant differences were found in vacF–IFN-γ- and vvGwt–IL-4-infected mice (P = 0.04 and P = 0.03, respectively). Thus, depending upon the foreign gene expressed, host factors such as deficiencies in immune mediators, IFN-γ-producing capacity, IFN-γ-induced signalling or the MHC phenotype may affect vaccinia virus titres at the site of infection.

Modulation of disease by co-expression of IFN-γ during vvGs immunization

Mice were immunized with vac-lac, vvGs or vvGs–IFN-γ and, 6 weeks later, were challenged with RSV. The mice were weighed and scored for illness for 7 days. Mice immunized with vvGs–IFN-γ exhibited significantly less illness after challenge than did vvGs-primed mice (Fig. 2a; P < 0.05), although no significant difference was observed between weight loss in vvGs- and vvGs–IFN-γ-primed mice (Fig. 2b). RSV titres were also similar in vvGs- and vvGs–IFN-γ-primed mice (Table 3). Thus, the immune responses induced during vvGs immunization that result in more severe disease upon RSV challenge may be partially regulated by IFN-γ expression in the priming microenvironment. In addition, despite lower vector titres at immunization, a degree of protection against RSV G-induced disease was maintained against subsequent RSV challenge, as evidenced by decreased illness and virus titres.

Co-expression of IFN-γ during vvGs priming results in a trend towards reduced BAL eosinophilia following RSV challenge

Lack of IFN-γ increases the magnitude of eosinophil-recruiting immune responses induced by RSV G (Srikitkachorn & Braciale, 1997b; Russell et al., 1997). The impact of IFN-γ co-expression during immunization on eosinophil recruitment into the airways was examined by BAL. Seven days after challenge, mice immunized with vvGs–IFN-γ had a trend towards smaller numbers of eosinophils than vvGs-primed mice (Table 3), although significant eosinophilia still occurred in vvGs–IFN-γ-primed mice (P = 0.01, comparing vac-lac- and vvGs–IFN-γ-primed mice).

Co-expression of IFN-γ during vvGs immunization does not alter cytokine production significantly following RSV challenge

IL-4, IL-5, IFN-γ and eotaxin were measured by ELISA using lung supernatants obtained 4 days after challenge. Immunization with vvGs or vvGs–IFN-γ resulted in significant production of IL-4, IL-5 and IFN-γ following RSV infection (Table 3; P ≤ 0.02, comparing vvGs- or vvGs–IFN-γ-primed mice to vac-lac-primed controls). However, IL-4, IL-5 and IFN-γ levels were similar in vvGs- and vvGs–IFN-γ-immunized, RSV-challenged mice. Immunization with vvGs resulted in
Table 3. Impact of IFN-γ co-expression on RSV G-induced immune responses

BALB/c mice were immunized and challenged as described in Fig. 2. RSV titres are represented as means ± SEM for ten mice combined from two separate experiments. The limit of detection is 1·5 log_{10}(p.f.u./g lung). Cytokine levels were measured by ELISA using lung supernatants obtained 4 days after RSV challenge. Data represent means ± SEM from ten mice combined from two separate experiments.

<table>
<thead>
<tr>
<th>Priming virus</th>
<th>RSV titre [log_{10}(p.f.u./g)]</th>
<th>Cytokine concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td>vac-lac</td>
<td>5·69 ± 0·32</td>
<td>2·62 ± 0·11</td>
</tr>
<tr>
<td>vvGs</td>
<td>4·91 ± 0·25</td>
<td>1·62 ± 0·07</td>
</tr>
<tr>
<td>vvGs–IFN-γ</td>
<td>5·43 ± 0·28</td>
<td>1·82 ± 0·17</td>
</tr>
</tbody>
</table>

* Data are percentages of total cells from BAL, represented as means ± SEM for ten mice combined from two separate experiments. Numbers of eosinophils (× 10^{3}) are given in parentheses.

Table 4. Impact of IFN-γ co-expression on antibody responses

Sera were collected 6 weeks after immunization and the day before RSV challenge (pre-challenge) and again 6 weeks after challenge (post-challenge). Data are represented as mean ± SEM log_{10}(serum dilution resulting in an A_{550} of 0·1) for ten mice pooled from two separate experiments. The limit of detection is 4·0. Titres of neutralizing antibody to vaccinia virus were measured in pre-challenge sera by plaque-reduction assay. Data represent mean ± SEM log_{10}(serum dilution resulting in 40% reduction) for ten mice pooled from two experiments.

<table>
<thead>
<tr>
<th>Priming virus</th>
<th>Pre-challenge titre</th>
<th>Post-challenge titre</th>
<th>Anti-vaccinia virus neutralizing titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total IgG</td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
<tr>
<td>vac-lac</td>
<td>4·10 ± 0·06</td>
<td>4·77 ± 0·56</td>
<td>4·00 ± 0·00</td>
</tr>
<tr>
<td>vvGs</td>
<td>11·08 ± 0·54</td>
<td>8·76 ± 0·97</td>
<td>6·35 ± 0·82</td>
</tr>
<tr>
<td>vvGs–IFN-γ</td>
<td>9·48 ± 0·32</td>
<td>5·04 ± 0·54</td>
<td>4·26 ± 0·14</td>
</tr>
</tbody>
</table>

eotaxin production after RSV challenge that was significantly greater than that in vac-lac-primed mice (P = 0·02). While eotaxin levels in vvGs–IFN-γ-primed, RSV-challenged mice were higher than in vac-lac-primed mice, the difference was not statistically significant (P = 0·17). Similarly, the decrease in eotaxin production in vvGs–IFN-γ-primed mice relative to vvGs-immunized mice was not significant (P = 0·15).

Titres of G-specific total IgG in vvGs- and vvGs–IFN-γ-primed mice were significantly greater than in vac-lac-primed mice in pre-challenge sera (P < 0·03). Levels of G-specific total IgG and immunoglobulin with an IgG2a isotype were similar in vvGs- and vvGs–IFN-γ-immunized mice in both pre- and post-challenge sera (Table 4; P ≥ 0·07 for total IgG and for IgG2a). However, titres of antibodies having an IgG1 isotype in pre-challenge sera were significantly higher in vvGs-primed mice than in vvGs–IFN-γ-immunized mice (P = 0·02). In contrast, in post-challenge sera, anti-G IgG1 titres were similar in these two groups of mice (P = 0·88). Thus, since the total IgG titres of G-specific antibody were not significantly different in pre-challenge sera of vvGs- and vvGs–IFN-γ-primed mice and were significantly higher than in vac-lac-primed mice, these data indicate that the G immunogenicity was maintained despite lower replication of the vvGs–IFN-γ vector, although there were non-significant reductions relative to the vvGs vector.

IFN-γ co-expression during vvGs priming does not decrease production of vaccinia virus-neutralizing antibodies, but does limit vaccinia virus replication

In order to compare the ability of vvGs and vvGs–IFN-γ to replicate and disseminate in vivo, mice were infected intradermally at the base of the tail and tails, ovaries and lungs were harvested. No virus was detected in ovaries or lungs of either vvGs- or vvGs–IFN-γ-infected mice. Therefore, IFN-γ did not increase dissemination of vaccinia virus within the limits of the assay. When vaccinia virus titres in the tail were examined over
were infected intradermally with vvGs (shaded bars) or vvGs–IFN-γ (filled bars) at the base of the tail and vaccinia virus titres were measured by plaque assay at the indicated times post-infection. Vaccinia virus titres at days 7, 10 and 14 post-infection were significantly reduced by IFN-γ co-expression (P < 0.03). Data are represented as means ± SEM log_{10}(p.f.u./g tail tissue) for four mice.

Fig. 3. IFN-γ co-expression during vvGs priming limits vaccinia virus replication. Mice were primed and challenged as described in Fig. 2. Mice were primed and challenged as described in Fig. 2. Mice were primed and challenged as described in Fig. 2. mice (% P07 log_{10}(p.f.u./g) in vvGs–IFN-γ–infected mice (P = 0.03). With dramatically lower virus titres, vvGs–IFN-γ was cleared from the tail by day 14, while 4.6 log_{10}(p.f.u./g) remained in vvGs-infected mice. Thus, co-expression of IFN-γ inhibited replication of the vaccinia virus vector and may therefore limit the production of antigen. Additionally, co-expression of IFN-γ reduced vvGs replication (Fig. 3, 1000-fold decrease) to a greater extent than seen with vvGwt (Table 2, 100-fold decrease).

Vaccinia virus replication was measured indirectly by the production of neutralizing anti-vaccinia virus antibodies and directly by plaque assay. Titres of vaccinia virus-neutralizing antibody were similar in vvGs- and vvGs–IFN-γ–primed mice (Table 4), suggesting that the threshold for induction of vaccinia virus-specific antibody was achieved despite attenuated growth of the vvGs–IFN-γ vector.

Discussion

The detailed characterization of the vaccinia virus genome and immune responses to vaccinia virus provides a mechanism for the construction of recombinant viruses expressing foreign genes. While replication-competent vaccinia viruses with full virulence may not be realistic candidates for vaccine vectors, they are important research tools for understanding pathogenesis and immunity (Baumgarth & Kelso, 1996). With such tools, vaccine efficacy and safety may be optimized by rational vector design. One approach to increasing vaccine efficacy is the insertion of genes for immune mediators to direct T-cell differentiation towards protective and disease-sparing effector pathways. Since induction of either type 1 or type 2 T-cell responses has been associated differentially with protective immune responses (Lucey et al., 1996), inclusion of a cytokine in a vaccine vector may potentially direct immune responses towards a more protective, less deleterious phenotype (Tang & Graham, 1996). However, the impact of vector-produced cytokine on replication of the vector and expression of antigen must be defined.

RSV disease in mice is primarily immune-mediated rather than a result of direct virus cytopathicity (Graham et al., 1991; Graham, 1995; Tang & Graham, 1997; Piedra et al., 1989). In order to evaluate the influence of cytokines on the antigenicity of RSV proteins, recombinant vaccinia virus vectors were constructed that co-expressed either IL-4 or IFN-γ and either RSV F or G. Prior to use in vaccination experiments, the growth characteristics of the recombinant viruses were compared. These studies demonstrated that co-expression of IFN-γ limits vaccinia virus replication in BALB/c mice independently of the recombinant co-expressed virus antigen, while the impact of IFN-γ co-expression on vaccinia virus replication is variable in C57BL/6 mice. These data underscore the pivotal influence that genetic background and host factors can have on the induction of distinct T-cell subsets.

Our data confirm earlier reports that demonstrated the ability of IL-4 to delay virus clearance and inhibit CTL generation (Sharma et al., 1996; Fischer et al., 1997; Bembridge et al., 1998b; Aung et al., 1999), in that IL-4 expressed by the vac-lac or vvGwt vectors resulted in higher virus titres. In contrast to the results reported by Bembridge et al. (1998b), when IL-4 was co-expressed with RSV F, a protein strongly associated with the induction of Th1 T cells, we did not see increased vaccinia virus titres. A potential explanation for this difference is the level of IL-4 expression. Bembridge et al. (1998b) constructed vaccinia viruses expressing both low and high levels of cytokine, but reported vaccinia virus replication only for vectors expressing high levels of IL-4. IL-4 production from this construct was more than 500-fold greater than the IL-4 levels produced by the recombinant virus in our report. We have extended the findings of earlier reports, which focused on single viral proteins in single mouse strains (Sharma et al., 1996; Bembridge et al., 1998b; Aung et al., 1999), and defined the influence of co-expressed cytokines on the immunogenicity of multiple RSV proteins (F, wild-type G and secreted G) in multiple strains of mice. We have demonstrated that the viral antigen and the mouse strain can alter the impact of the co-expressed cytokine. When the ability of virus-expressed IL-2 to accelerate clearance of vaccinia virus was examined in three different strains of primates, the extent of attenuation was not uniform (Flexner et al., 1990). Similarly, in mice immunized with vaccinia virus-expressed RSV G, the degree of pulmonary eosinophilia after RSV challenge was influenced by the MHC haplotype of the mouse strain (Hussell et al., 1998). Thus, the impact of cytokine co-expression is modulated by the recombinant antigen co-expressed in the vector and by the
genetic composition of the host. Therefore, both host and vector factors must be considered in the design of immunomodulatory immunization protocols.

The immunomodulatory capacity of vaccine-expressed cytokines becomes important in settings of apparently ineffective immunization, such as with vacF. While vacF has been shown to protect rodents against RSV challenge (Levine et al., 1989; Murphy et al., 1989; Olmsted et al., 1986), this immunization strategy failed to protect non-human primates against subsequent RSV infection (Olmsted et al., 1988). Yet, the immunization did affect the induction of immune responses, as evidenced by significant increases in neutralizing antibody titres in vacF-immunized monkeys following RSV challenge. These data indicate the potential of immunization strategies using RSV F protein, yet demonstrate that, in primates, an effective vaccination protocol will require additional immunomodulation (either during initial immunization or as an antigenic boost) to achieve immunity against RSV challenge.

IFN-γ production, by CD8+ CTL (Srikiatkhachorn & Braciale, 1997b; Hussell et al., 1997b) or by NK cells (Hussell & Openshaw, 1998), has been shown to reduce eosinophilia induced by RSV G. We therefore hypothesized that co-expression of IFN-γ during vvGs immunization would modulate G-specific immune responses that augment disease upon RSV challenge. We found that vvGs replication was attenuated in the presence of IFN-γ, but immunogenicity was preserved. This is similar to findings obtained when RSV itself was used to co-express IFN-γ, resulting in attenuated virus growth with no reduction in immunogenicity (Bukreyev et al., 1999). However, this is in contrast to the data of Bembridge et al. (1998b), which demonstrated a reduction in antibody titres in mice immunized with vaccinia virus co-expressing RSV F and IFN-γ. Although not statistically significant, there was a trend towards reduced eosinophilia and eotaxin production in vvGs–IFN-γ-primed mice (relative to vvGs-primed controls). Since IFN-γ co-expression limited replication of the vvGs–IFN-γ vector and thus limited production of G, the reduced capacity of vvGs–IFN-γ-induced immune responses to recruit eosinophils may be the result of an altered magnitude of response rather than an altered composition of immune effectors. Yet, it must be noted that the immune responses generated during vvGs immunization occur in the presence of significant IFN-γ production resulting from vaccinia virus infection. While previous work suggests that IFN-γ production during RSV G immunization modulates eosinophil-inducing immune responses, these data suggest that RSV G stimulates factors in addition to IFN-γ that contribute to the induction of immune responses, resulting in eosinophil activation and recruitment upon subsequent RSV challenge. In addition, we have shown that vvGs-induced eosinophilia and IL-13 production occur in the absence of IL-4 in both BALB/c and C57BL/6 mice (Johnson & Graham, 1999). Thus, in the presence of IFN-γ expression induced by vaccinia virus immunization and in the genetic background of C57BL/6 mice, RSV G may still induce eosinophilia. Recent reports provide a potential explanation for these data, as they demonstrate that the immunodominant epitope of RSV G induces both Th1 and Th2 cytokine expression (Sparrer et al., 1998; Srikiatkhachorn et al., 1999; Tebbey et al., 1998; Varga et al., 2000). Varga et al. (2000) went on to show that G-specific CD4+ T cells express a restricted set of TCR Vβ genes, with Vβ14 predominant. These observations provide a potential molecular mechanism for the ability of RSV G immunization, in the presence of vaccinia virus-induced IFN-γ, to predispose for Th2-oriented immune responses. Furthermore, these data provide an explanation for the inability of vvGs–IFN-γ to fully modulate G-induced eosinophilia and eotaxin production. By co-expressing IFN-γ in the priming microenvironment, we may modulate the classical Th2 component of RSV G-induced responses, resulting in partial (though not statistically significant) changes. However, IFN-γ co-expression is unable to regulate the non-classical Th2 component of RSV G immunopathogenesis contributed by this Vβ14-expressing T-cell subset. Together, these data underscore the complexity of RSV G immunopathogenesis and provide a molecular explanation for the ability of RSV G to predispose for type 2 immune responses in the presence of IFN-γ production, a contradiction of the classical Th1–Th2 paradigm.

We have produced recombinant vaccinia viruses that co-express individual RSV proteins and murine cytokines. Characterization of these vectors demonstrates that IFN-γ has promise as an immunomodulator for vaccine delivery. However, while co-expression of immune mediators may serve to direct T-cell differentiation towards safer phenotypes, virus replication may also be altered, thereby limiting antigen load and the overall immunogenicity of the immunization protocol. We have shown that the effect of cytokine co-expression is influenced by both the RSV antigen and the genetic background of the mouse strain, suggesting that different vaccine formulations may be required for effective immunization of the distinct at-risk human populations. In addition, we demonstrate that the timing of immunization relative to challenge or natural infection is important. In order to evaluate fully the capacity of cytokine-expressing recombinant viral vectors to alter immune responses to subsequent challenge, a sufficient interval must be allowed for the resolution of the primary immune responses induced by the vector.

This work was supported by National Institutes of Health grants R01-AI-37216 and R01-AI-33933. We gratefully acknowledge the technical assistance of Rauf Kuli-Zade and Robert A. Parker (Department of Medicine [Biostatistics], Harvard University) for the initial development of the SAS programs used for statistical analysis.

References


B-cell immunity to respiratory syncytial virus induced by individual viral proteins. *Vaccine* 11, 431–437.


Bembridge, G. P., Lopez, J. A., Cook, R., Melero, J. A. & Taylor, G. (1998b). Recombinant vaccinia virus coexpressing the F protein of respiratory syncytial virus (RSV) and interleukin-4 (IL-4) does not inhibit the development of RSV-specific memory cytotoxic T lymphocytes, whereas priming is diminished in the presence of high levels of IL-2 or gamma interferon. *Journal of Virology* 72, 4080–4087.


Received 2 February 2001; Accepted 6 June 2001