DNA Vaccination with Vaccinia Virus L1R and A33R Genes Protects Mice against a Lethal Poxvirus Challenge

J. W. Hooper,1 D. M. Custer, C. S. Schmaljohn, and A. L. Schmaljohn

Virology Division, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, Maryland 21702

Received October 5, 1999; returned to author for revision November 11, 1999; accepted November 17, 1999

Previously we found that passive transfer of monoclonal antibodies (MAbs) specific to either the vaccinia virus (VACV) L1R or A33R gene product protected mice from challenge with VACV. The L1R-specific MAbs, which bind the intracellular mature virion (IMV), neutralized virus in cell culture, whereas the A33R-specific MAbs, which bind extracellular enveloped virions (EEV), did not. To investigate whether a protective response could be generated by vaccination with these genes, we constructed and evaluated DNA vaccines expressing the VACV L1R and/or A33R genes under control of a cytomegalovirus promoter. Mice were vaccinated with DNA-coated gold beads by using a gene gun and then challenged with VACV (strain WR) intraperitoneally. Mice vaccinated with L1R alone developed neutralizing antibodies and were partially protected. Mice vaccinated with a combination of both genes loaded on the same gold beads developed a robust anti-A33R response; however, no neutralizing antibody response was detected, and the mice were not protected. In contrast, when mice were vaccinated with L1R and A33R loaded on different gold beads, neutralizing (presumably anti-L1R) and anti-A33R antibody responses were detected, and protection was markedly improved. Our results indicated that vaccination with both L1R and A33R proteins, intended to evoke mechanistically distinct and complementary forms of protection, was more effective than vaccination with either protein by itself.

INTRODUCTION

Viruses in the family Poxviridae, including vaccinia virus (VACV) and variola virus, are characterized by a large linear double-stranded DNA genome (130–300 kb) packaged in a relatively large virion (~350 × 270 nm), and a cytoplasmic site of replication (reviewed by Moss, 1996). Assembly of VACV virions begins with condensation of dense granular material into membrane-wrapped particles called intracellular mature virions (IMV). Recent findings indicate the IMV are wrapped by a single membrane (Hollingshead et al., 1999) rather than a double membrane as previously reported. IMV are then enveloped in two additional membranes derived from the trans Golgi to form multiple membrane-wrapped particles called intracellular enveloped virions (IEV) (Schmelz et al., 1994). IEV are moved, possibly by actin polymerization (Cudmore et al., 1995), to the cell periphery, where the outermost membrane fuses with the cell plasma membrane, exposing a cell-associated enveloped virion (CEV) (Blasco and Moss, 1991). CEV are released from the cell as extracellular enveloped virions (EEV), which play a role in long-range spread of the virus (Payne, 1980). IMV released from disrupted cells and EEV are both infectious forms of VACV.

To identify potential targets for poxvirus vaccines or therapeutics, we previously generated and characterized a panel of VACV-specific monoclonal antibodies (MAbs) (unpublished data). Passive protection experiments in mice indicated that neutralizing MAbs binding a 29-kDa protein (e.g., MAb-10F5, MAb-7D11), and nonneutralizing MAbs binding a 23- to 28-kDa protein (e.g., MAb-1G10) protected against challenge with VACV (strain WR) (unpublished data). The target of MAb-7D11 was the product of the L1R gene (Wolffe et al., 1995), and the target of MAb-1G10 was the product of the A33R gene (Roper et al., 1996, and personal communication). In this report, the L1R and A33R gene products will be called L1R and A33R, respectively. L1R is an essential myristoylated protein associated with the IMV membrane and is thought to play a role in IMV attachment or penetration (Franke et al., 1990; Ravanello et al., 1993; Ichihashi et al., 1994; Ravanello and Hruby, 1994; Wolfe et al., 1995). A33R is a nominally nonessential glycosylated/palmitated protein that forms dimers and is incorporated into the outer membrane of EEV (Payne, 1992; Roper et al., 1996). A33R is thought to be involved in facilitating direct cell-to-cell spread via actin-containing microvilli (Roper et al., 1998). Homologs of L1R and A33R are present in other orthopoxviruses; between VACV and variola, L1R identity is 99.6% and A33R is 94.1% (Massung et al., 1994).

Naked DNA vaccines have been used to generate protective immune responses against numerous pathogenic agents, including many viruses (Gregoriadis,
1998). In general, naked DNA vaccines involve vaccination with plasmid DNA that contains a gene of interest controlled by a cytomegalovirus (CMV) promoter. When the plasmid is introduced into mammalian cells, cell machinery transcribes and translates the gene. The expressed protein (immunogen) is then presented to the immune system where it can elicit an immune response. One method of introducing DNA into cells is by using a gene gun. This method of vaccination involves using pressurized helium gas to accelerate DNA-coated gold beads into the skin of the vaccinee.

To determine whether vaccination with the L1R and/or A33R gene could elicit protective immunity, we constructed plasmids expressing either L1R or A33R under control of the CMV promoter and tested these plasmids, and combinations of these plasmids, for immunogenicity and protective efficacy in mice. Our results indicated that vaccination with both L1R and A33R proteins, when loaded on different gold beads and hence delivered to different cells, was more effective than vaccination with either protein by itself.

RESULTS

Cloning the vaccinia A33R and L1R genes into a naked-DNA vector and transient expression in cell culture

The A33R and L1R genes from VACV (Connaught vaccine strain) were PCR amplified and cloned into a naked-DNA expression vector pWRG7077 (Schmaljohn et al., 1997) to yield constructs pWRG/A33R and pWRG/L1R, respectively (Fig. 1). Sequence analysis of the L1R and A33R clones indicated that the Connaught strain and WR strain genes are identical at the amino acid level.

To determine whether the appropriate gene products were expressed, pWRG/L1R or pWRG/A33R were transfected into COS cells, and radiolabeled proteins were immunoprecipitated with MAbs specific to each protein. MAb-1G10 (A33R-specific) immunoprecipitated a product with an apparent size of 23–28 kDa under reducing conditions and 55 kDa under nonreducing conditions (Fig. 1B). MAb-10F5 (L1R-specific) immunoprecipitated products with apparent sizes of 25 and 29 kDa under reducing and nonreducing conditions (Fig. 1C). Under reducing conditions, the predominant product ran at an apparent size of 29 kDa, and under nonreducing conditions, the predominant product ran at an apparent size of 25 kDa. Thus both pWRG/A33R and pWRG/L1R expressed proteins that were bound by A33R- and L1R-specific MAbs and had predicted electrophoretic mobilities.

Vaccination with pWRG/L1R elicits neutralizing antibody responses in mice

To determine whether vaccination with pWRG/L1R or pWRG/A33R elicited antibody responses in mice, groups of 9–10 mice were vaccinated with pWRG/L1R, pWRG/A33R, a combination of pWRG/L1R and pWRG/A33R on the same gold beads (pWRG/L1R+pWRG/A33R[same gold]), or a negative control plasmid (pWRG7077) (Experiment 1, Table 1). As positive controls, 10 mice were vaccinated by tail scarification with VACV (Connaught strain). Sera were collected before initial vaccinations (prebleed) and 12 weeks after the final boost.

To measure L1R-specific antibody responses, we performed plaque-reduction neutralization tests (PRNT). All 10 mice vaccinated with pWRG/L1R produced VACV-specific neutralizing antibodies exhibiting PRNT titers ranging from 80 to 320, geometric mean titer (GMT) = 197. Likewise, all 10 mice scarified with VACV produced neutralizing antibodies with titers ranging from 80 to 1280, GMT = 368.

Neutralizing antibodies were not detected in prebleeds or in sera from mice vaccinated with either pWRG/A33R or pWRG7077. Interestingly, neutralizing antibodies were not detected in any of nine mice vaccinated with pWRG/L1R+pWRG/A33R[same gold]. Mean PRNT values for vaccinated mice sera, and control antibodies are shown (Fig. 2). Thus a neutralizing antibody response was elicited when mice were vaccinated with pWRG/L1R but not when pWRG/L1R was combined on the same gold beads as pWRG/A33R.

Vaccination with pWRG/A33R elicits antibody responses in mice

To measure A33R-specific antibody responses, we developed an ELISA that uses a fixed cell monolayer, previously transfected with pWRG/A33R, as the solid-phase antigen. This ELISA is based on the observation that cells transfected with the A33R gene, or infected with VACV, exhibit a strong signal when immunostained with A33R-specific MAbs (Roper et al., 1996). All 10 mice vaccinated with pWRG/A33R exhibited an anti-A33R antibody response with titers ranging from 400 to 6400, GMT = 1600. Similarly, nine of nine mice vaccinated with pWRG/A33R+pWRG/L1R[same gold] exhibited an anti-A33R antibody response with titers ranging from 400 to 6400, GMT = 2352. Only 4 of 10 scarified mice exhibited detectable anti-A33R antibody responses with titers ranging from 200 to 800, GMT = 174. Mean ELISA values for vaccinated mice and control antibodies are shown (Fig. 3). Positive control antibodies, MAB-1G10 and VACV HMAF, had titers of 6400 and 1600, respectively. A second anti-A33R antibody, MAB-10F10, had a titer of 3200. Thus vaccination with pWRG/A33R alone or in combination with pWRG/L1R elicited a nonneutralizing antibody response in mice that was significantly greater than the anti-A33R response elicited by tail scarification with live VACV. This result was reproduced in three separate experiments (data not shown). Moreover, although an anti-L1R response was undetected in mice vaccinated with...
FIG. 1. Naked DNA constructs expressing VACV L1R or A33R genes. (A) The L1R and A33R genes from VACV (Connaught strain) were PCR-amplified and cloned into a naked DNA expression vector pWRG7077 to yield constructs pWRG/L1R and pWRG/A33R, respectively. The VACV genes are flanked by a cytomegalovirus immediate early promoter (CMV IE) and intron A at the 5’ end of the gene and a bovine growth hormone polyadenylation signal (BGH pA) at the 3’ end. Kanamycin antibiotic resistance gene (KAN). (B) Expression products from COS cell monolayers transfected with pWRG/A33R, or mock transfected, were immunoprecipitated with MAb-1G10. Samples were boiled in reducing or nonreducing sample buffer, and separated by SDS–PAGE. (C) Expression products from COS cell monolayers transfected with pWRG/L1R, or mock transfected, were immunoprecipitated with the L1R-specific antibody MAb-10F5. Samples were boiled in reducing or nonreducing sample buffer, and separated by SDS–PAGE. Molecular mass markers in kDa are shown at right of each gel.
pWRG/L1R and/or pWRG/A33R could protect mice from lethal challenge with VACV, mice were vaccinated with a single construct or both constructs and then challenged with a lethal dose of VACV. Dual construct vaccinations were performed with either a combination of both plasmids on the same gold beads or gold beads coated with individual constructs. The vaccination schedule of Experiment 2 is shown in Table 1.

Most mice vaccinated with pWRG/L1R developed neu-

### TABLE 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Immunogen</th>
<th>DNA per cartridge (µg)</th>
<th>Number of cartridges per dose</th>
<th>Boost 1 (wks after priming)</th>
<th>Boost 2 (wks after boost 2)</th>
<th>Final bleed (wks after final boost)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(−)control</td>
<td>pWRG/L1R</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>pWRG/A33R</td>
<td>pWRG/L1R+pWRG/A33R (same gold)</td>
<td>0.5 each</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Scarification</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(−)control</td>
<td>pWRG/L1R</td>
<td>0.5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>pWRG/A33R</td>
<td>pWRG/L1R+pWRG/A33R (same gold)</td>
<td>0.5 each</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Scarification</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** NA, not applicable.

*(−)control, negative control plasmid.

VACV (Connaught strain) (10 µl drop of PBS containing $8 \times 10^{5}$ PFU scratched into tail −1 cm from base) as positive controls.

FIG. 2. VACV-neutralizing antibody response elicited by gene gun vaccination with pWRG/L1R. Mean PRNT values of 10 mice vaccinated with pWRG/L1R, or 10 mice scarified with VACV are shown. Also shown are the mean PRNT values of groups of 10 mice vaccinated with pWRG/A33R, pWRG/A33R plus pWRG/L1R combined on the same gold beads, or a negative control plasmid. Titrations of positive control antibody ascitic fluid, MAb-7D11 and VACV HMAF, are also shown.
tralizing antibodies (Groups 2, 5, 7, and 8; Fig. 4); how-
ever, as in Experiment 1, if pWRG/A33R was combined on
the same gold beads with pWRG/L1R, none of the mice
developed neutralizing antibodies (Group 4, Fig. 4). Most
mice vaccinated with pWRG/A33R developed anti-A33R
antibodies regardless of whether pWRG/L1R was
present on the same gold beads (Groups 3, 4, 6, 7, and 9;
Fig. 4). All of the scarified mice developed neutralizing
antibodies, which represent not only anti-L1R antibodies
but also antibodies to other neutralizing antigens. Only
one scarified mouse in Experiment 2 had an anti-A33R
titer $200 (Group 10, Fig. 4).

Two weeks after the final vaccination, mice were chal-
lenged intraperitoneally (i.p.) with $5 \times 10^8$ PFU (12.5 LD$_{50}$)
of VACV WR. The results of the protection experiment are
shown in Figs. 4 and 5. All mice vaccinated by tail
scarification survived challenge with minimal clinical
signs of disease except a transient weight loss (Fig. 5).
All mice vaccinated with the negative control plasmid
died within 3 days (Fig. 5A). Most mice vaccinated with
pWRG/L1R alone (Group 2), or combined with a negative
control plasmid, on the same or different gold (Groups 5
and 8), survived challenge, suggesting vaccination with
pWRG/L1R provided partial protection against an i.p.
challenge with VACV WR. In most cases, the L1R-vacci-
nated mice that succumbed did so at later times after
challenge than controls (Fig. 5A). Although seven of nine
mice vaccinated with pWRG/A33R alone survived chal-
lence (Group 3), none of the mice vaccinated with pWRG/
A33R combined with a negative control plasmid, on the
same or different gold, survived challenge (Groups 6 and
9). Mice vaccinated with pWRG/A33R alone, that sur-
vived challenge, exhibited sustained morbidity (a greater
than 10% reduction in body weight on Days 2–5 post
challenge) (Fig. 5B). In a follow-up experiment performed
to further examine the protective efficacy of vaccination
with pWRG/A33R alone, 10 of 10 mice died within 4 days
despite having anti-A33R antibody titers comparable to
those in Group 3, Fig. 4 (data not shown). Together these
data suggest that vaccination with A33R provided mini-
mal protection against i.p. challenge with VACV WR and
was not an effective vaccine against i.p. challenge.

When mice were vaccinated with both plasmids, the
results differed dramatically depending on whether the
plasmids were loaded on the same or different gold
beads. When pWRG/A33R and pWRG/L1R were com-
bined on the same gold beads, all but one of the mice
died (Group 4). In contrast, when the plasmids were
loaded on different gold beads all of the mice were
protected (Group 7). Morbidity, as measured by weight
loss, was similar in the scarified mice and the mice
vaccinated with pWRG/L1R+pWRG/A33R[different gold]
(Fig. 5B).

DISCUSSION

The smallpox vaccine, i.e., scarification with VACV, is
one of the oldest and most successful vaccines ever
developed. A sustained worldwide vaccination program
resulted in the eradication of naturally occurring small-
pox disease in 1979. However, elimination of smallpox
has not eliminated the need for a poxvirus vaccine and/or
other anti-poxvirus measures such as vaccinia
immunglobulin (VIG) because other pathogenic poxvi-
ruses (e.g., monkeypox virus) and bioterrorism remain a
threat (Breman and Henderson, 1998). We are interested
in determining if a subset of VACV genes administered as naked DNA constructs could provide protection against poxvirus infection (i.e., replace VACV scarification) and/or serve as immunogen for the generation of protective monoclonal antibodies (i.e., replace VIG).

Of the ~200 genes that comprise the vaccinia ge-

---

**FIG. 4.** Prechallenge antibody titers and survival data. Sera from mice vaccinated as described in Table 1, Experiment 2, were evaluated for anti-L1R activity by PRNT, and for anti-A33R activity by ELISA. Sera were collected immediately before challenge. PRNT and ELISA titers for individual mice in each group are shown. Filled bars represent animals that did not survive challenge, and cross-hatched bars represent survivors. For the scarified mice (Group 10), the PRNT values represent all VACV neutralizing antibodies, not just the anti-L1R response. In groups where positive antibody responses were detected, geometric mean titers (GMT) are shown. NT, not tested.
nome, only 5 encode proteins that are known to elicit a neutralizing antibody response including: H5R (Gordon et al., 1991), A27L (Rodriguez and Esteban 1987; Lai et al., 1991), B5R (Galmiche et al., 1999), D8L (Hsiao et al., 1999), and L1R (Ichihashi et al., 1994; Wolfe et al., 1995).

Given the structural complexity of VACV, there may be...
other neutralizing antigens not yet identified. In addition, the A33R gene encodes a protein that elicits a nonneutralizing antibody response that is, nevertheless, protective (Galmiche et al., 1999; Schmaljohn, unpublished data). Two core proteins (A10L and A4L) demonstrate some protective immunity; however, it is unclear if the immunity is humoral or cell-mediated (Demkowicz et al., 1992). As a first step toward determining if a combination of vaccinia proteins could provide the basis for an alternative poxvirus vaccine or VIG, we evaluated the immunogenicity and protective efficacy of two gene products that are found either on the IMV or EEV.

L1R

Our results show that vaccination with the L1R gene can elicit neutralizing antibodies and provide protection against lethal poxvirus infection. We found that the monospecific neutralizing antibody titers elicited by vaccination with L1R were only twofold lower than the polyclonal neutralizing antibody response generated by tail scarification with VACV. We suspect that modification of the clonal neutralizing antibody response generated by vaccination with L1R were only twofold lower than the polyclonal neutralizing antibody titers elicited by vaccination with VACV. We found that the IMV released from infected cells (by immune mechanisms or by virus-induced cell lysis). We have not investigated the possibility that vaccination with L1R elicits a cell-mediated immune response that contributes to protective immunity.

A33R

Based on our earlier passive protection studies of MAbs specific to A33R, we suspected that vaccination with a naked DNA construct expressing the A33R product might confer protection. The experiments reported here, and those recently reported by others (Galmiche et al., 1999), confirmed that vaccination with the A33R gene does provide some protection against VACV. Galmiche et al. found that vaccination of mice with purified baculovirus expressed A33R protein, or a naked DNA construct expressing A33R, protected against a lethal intranasal (i.n.) VACV (strain IHD-J) challenge (Galmiche et al., 1999). In addition, Galmiche et al. found that passive transfer of serum from A33R vaccinated mice, but not rabbits, could passively protect mice against i.n. challenge. We previously found that A33R-specific monoclonal antibodies (e.g., MAb-1G10) plus complement lysed VACV-infected cells (Schmaljohn, unpublished data). Together these findings suggest that vaccination with A33R elicits antibodies that provide a degree of protection by directing the lysis of VACV-infected cells. In our challenge model, i.p. challenge with VACV WR, vaccination with A33R alone protected some mice in one experiment (Group 3 in Figs. 4 and 5); however, only 1 of 10 mice was protected when A33R was combined with L1R on the same gold beads, and none of the mice vaccinated with A33R combined with a negative control plasmid were protected despite relatively high anti-A33R antibody titers. Also, in a follow-up experiment designed to reexamine the protective efficacy of A33R, all 10 mice vaccinated with pWRG/A33R alone died within 4 days after i.p. challenge. These data suggest that an anti-A33R response fails to confer a consistent level of protection against an i.p. challenge with VACV WR.

It seems likely that an immune response to A33R plays a role principally in reducing the dissemination of virus or the yield of infectious virions per cell not in preventing primary infection. Failure of vaccination with A33R to consistently protect mice from an i.p. challenge might indicate that the initial infection is itself lethal or levels of disseminating progeny virus produced after challenge with $5 \times 10^9$ PFU overwhelmed the anti-A33R immune response. On the other hand, vaccination with A33R may protect against a smaller challenge dose that requires more dissemination for lethality, such as the i.n. route of challenge used by Galmiche et al. (10$^{4-5}$ PFU of the IHD-J strain of VACV) (Galmiche et al., 1999).

L1R+ A33R[same gold]

Although mice vaccinated with both L1R and A33R on the same gold beads had anti-A33R responses equivalent to those given only A33R, neutralizing antibodies were not detected. To our knowledge, this is the first description of one DNA vaccine immunogen suppressing the antibody response to a codelivered immunogen. This result was not due to a technical problem in coloading two plasmid preparations on the same gold beads because both plasmids could be eluted from the cartridges used to vaccinate the mice (data not shown). In
addition, this result was not due to A33R- and L1R-specific antibody interaction (e.g., A33R-specific antibodies sterically interfering with binding of L1R-specific antibodies) because mice vaccinated with L1R and A33R on different gold exhibited high titers of both A33R-specific antibodies and neutralizing antibodies (presumably L1R-specific). Although there was no evidence that A33R affects L1R immunogenicity in VACV-infected cells, it is possible that A33R downregulated translation or processing of L1R in plasmid-transfected cells by direct or indirect interactions and in doing so suppressed L1R immunogenicity. Another possible explanation for our results is that A33R-specific antibodies, elicited during the first vaccination, may have directed lysis of A33R-expressing cells during subsequent boosts and, in doing so, diminished the boosting effect. This hypothesis predicts that immunogens that require boosts to elicit detectable immune responses may be adversely affected by codelivery of pWRG/A33R. Consistent with this hypothesis, neutralizing antibodies to VACV were not detected after a single vaccination with pWRG/L1R (data not shown), indicating boosts are required for a detectable anti-L1R response. It remains to be determined if pWRG/A33R can inhibit immune responses to other codelivered immunogens.

**L1R+A33R[different gold]**

Vaccination with L1R and A33R administered on different gold beads, and therefore delivered to different cells, resulted in a greater level of protection than either immunogen alone. Mice vaccinated with both immunogens appeared to be protected almost as well as the scarified mice. Our working hypothesis is that L1R-specific antibodies limit the initial infection by neutralizing challenge virus (which is predominantly IMV), and A33R-specific antibodies are involved in preventing EEV dissemination by eliminating EEV or infected cells (e.g., via antibody-dependent cell-mediated cytotoxicity or antibody-dependent complement-mediated cytotoxicity). Lysis of infected cells may result in release of IMV and, in the absence of IMV neutralizing antibodies, may allow IMV-mediated dissemination.

The challenge model used here, WR strain of VACV administered to mice by the i.p. route, has been used previously to assess the protective efficacy of vaccination with individual VACV immunogens (Demkowitz et al., 1992). We used this model to demonstrate that vaccination with L1R and A33R provides protection; however, because this is one vaccination protocol, one challenge virus, and one route of challenge, it will be important to evaluate the protective efficacy of these immunogens in other challenge models that use different viruses (e.g., other virulent VACV strains such as the IHD-J strain or other poxviruses such as monkeypox virus), different routes of administration (e.g., i.n. or aerosol routes), or different susceptible animal species (e.g., monkeys). It also will be necessary to optimize vaccine formulations and vaccination schedules. Information derived from alternative vaccine protocols and challenge models should help us better predict whether or not a combination of poxvirus immunogens (e.g., L1R and A33R), as a vaccine or source of immunoglobulin, might confer protection in humans.

In summary, in this study we demonstrated that vaccination of mice with VACV genes encoding proteins found on the surface of two infectious forms of the virus (L1R found on the IMV and A33R found on the EEV) provide a greater level of protection than vaccination with either gene alone. By combining additional VACV immunogens with L1R and A33R, it may be possible to develop a vaccine that elicits an even more potent and redundant anti-poxvirus immune response. These studies should also help identify targets for the rational design of a monoclonal antibody-based replacement for VIG.

**MATERIALS AND METHODS**

**Viruses and cells**

VACV Connaught vaccine strain (derived from the New York City Board of Health strain) (McClain et al., 1997) and strain WR (Western Reserve) (ATCC VR-119) were maintained in VERO cell (ATCC CRL-1587) monolayers grown in Eagle minimal essential medium, containing 5% heat-inactivated fetal bovine serum, 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin), 10 mM HEPES (cEMEM)s. COS cells (ATCC CRL 1651) were used for transient expression experiments.

**Antibodies**

Two L1R-specific (MAb-7D11 and MAb-10F5) and two A33R-specific (MAb-1G10 and MAb-10F10) Mabs, as mouse asctic fluids, were used. VACV (strain Connaught) hyperimmune mouse ascitic fluid (VACV HMAF) was also used.

**Cloning L1R and A33R into naked-DNA expression plasmids**

VACV (Connaught strain) DNA was purified by standard methods and used as template for PCR and cloning of the L1R and A33R genes. PCR primer design was based on the published VACV (Copenhagen strain) sequence (accession number M35027). The L1R primers were: 5′-gccgccgcctcattggttcgctcgaagctctcagt and 5′-gccgccgcctcattggttcgctcgaagctctcagt; and the A33R primers were: 5′-gccgccgcctcattggttcgctcgaagctctcagt and 5′-gccgccgcctcattggttcgctcgaagctctcagt. Not sites (underlined) were incorporated at gene termini. Start codons are shown in bold. L1R and A33R were
PCR-amplified using VENT polymerase (NEB), cut with NotI, and cloned into the NotI site of plasmid pWRG7077 (Schmaljohn et al., 1997) to yield naked DNA expression plasmids pWRG/L1R and pWRG/A33R, respectively.

**Transient expression**

Plasmid DNA was transfected into COS cell monolayers (60–80% confluent) using Lipofectin or Fugene6 reagent as described by the manufacturer. After 24–48 h, monolayers were radiolabeled with Promix (200 μCi per T-25 flask, [35S]methionine and [35S]cysteine; Amersham) and immunoprecipitated as follows. Transfected cells were lysed on ice for 5 min with a modified RIPA buffer: 0.25 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.4, and protease inhibitors (Complete; Boehringer Mannheim). To increase the yield of precipitable L1R, 10 mM iodoacetamide was included (Wolffe et al., 1995). Lysates were combined with protein A sepharose (CL-4B; Sigma), incubated overnight at 4°C. Lysate-antibody mixtures were combined with unlabeled COS cell lysate and incubated overnight at 4°C. Lysate-antibody mixtures were combined with protein A sepharose (CL-4B; Sigma), incubated at 4°C for 30 min, and then washed three times with lysis buffer and once with 10 mM Tris, pH 8.0. Sample buffer [125 mM Tris (pH 8.0), 1% SDS, 10% glycerol, 0.01% bromphenol blue containing 2% 2-mercaptoethanol for reducing gels or 10 mM iodoacetamide for nonreducing gels] was added and the samples were boiled for 2 min. Samples were then analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography.

**Vaccination with the gene gun**

Cartridges for the gene gun were prepared as described previously (Eisenbraun et al., 1993; Schmaljohn et al., 1997). Briefly, plasmid DNA was precipitated onto ~2 μm diameter gold beads (Degussa), 1 μg DNA per 1 mg gold, which were then coated on the inner surface of Tefzel tubing (McMaster-Carr). The tubing was cut into 0.5-in cartridges. When completed, each cartridge contained 0.25–0.5 μg of DNA coated on 0.5 mg of gold. To vaccinate animals, abdominal fur was removed with clippers and DNA-coated gold was administered to two nonoverlapping sites on the abdominal epidermis by using the gene gun (Powderject Delivery Device, Powderject, Inc.) at 400 p.s.i. as described previously (Pertmer et al., 1995).

**Plaque reduction neutralization assay**

VACV-infected cell lysate was diluted in cEMEM to give ~1000 PFU/ml. Aliquots of this virus suspension (100 μl) were incubated with an equal volume of antibody diluted in cEMEM (serum samples were heat inactivated, 56°C for 30 min, prior to dilution) for 1 h at 37°C and then 180 μl of sample was adsorbed to VERO cell monolayers in 6-well plates (or 12-well plates) for 1 h. A 2 ml cEMEM liquid overlay was added to each well (1 ml for 12-well plates). After 3 days at 37°C, monolayers were stained with 1% crystal violet dissolved in 70% ethanol. Plaques were counted and the percent neutralization was calculated relative to plaque numbers in the absence of antibody. Titers represent the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques.

**Transfection/ELISA method**

COS cell monolayers, grown in 96-well cell culture plates, were transfected with pWRG/A33R (0.2 μg/well) using Fugene6 or were mock transfected. After ~24 h the monolayers were fixed with 1:1 acetone–methanol for 2 min and immunostained as previously described (Roper et al., 1996); however, the dianisidine substrate was replaced with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate. Briefly, monolayers were fixed with 1:1 acetone–methanol for 2 min, rinsed with PBS, incubated 1 h with primary antibody diluted in PBS+3%FBS, rinsed with PBS, incubated 30 min with peroxidase-labeled goat anti-mouse antibody (Sigma) diluted in PBS+3%FBS, rinsed, and finally, incubated with ABTS. After ~30 min, 100 μl per well of 0.2 N phosphoric acid was added and the O.D. at 405 nm was determined by an ELISA plate reader. O.D. values from mock-transfected wells were subtracted from those of transfected wells to determine the specific O.D. 405 nm for each sample. End-point titers were determined as the highest dilution with an absorbence value greater than the mean absorbence value from negative control plasmid (pWRG7077)-vaccinated animals plus three standard deviations.

**Challenge experiment**

Mice were injected with 5 × 10⁸ PFU of VACV strain WR (12.5 LD₅₀) (clarified infected cell lysate) by the intraperitoneal route (i.p.) with a 0.5 × 16 mm needle. This research was conducted in accordance with procedures described in the Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1996). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**ACKNOWLEDGMENTS**

The gene gun (Powderject delivery device) and pWRG7077 were kindly provided by Powderject Vaccine, Inc.

**REFERENCES**


