

## Neutralizing and Protective Antibodies Directed against Vaccinia Virus Envelope Antigens

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The infection mechanism of vaccinia virus is largely unknown. Neither the attachment protein of extracellular enveloped virus (EEV), the biologically relevant infectious form of the virus, nor its cellular receptor has been identified. Surprisingly, all former attempts using antibodies to block EEV infection of cells *in vitro* had failed. Here, we report the production of an anti-envelope hyperimmune serum with EEV neutralizing activity and show that a polyclonal antiserum against the extraviral domain of protein B5R also inhibited EEV infection. *In vivo*, mice vaccinated with B5R protein were protected against a lethal vaccinia virus challenge. This protectivity is likely to be mediated by neutralizing antibodies. Protein A33R, but not A34R and A36R, also proved to be protective in active and passive vaccination experiments. However, in contrast to B5R, A33R protectivity did not correlate with antibody titers. Because anti-A33R antibodies did not neutralize EEV *in vitro*, the protectivity mediated by A33R protein probably involves a mechanism different from simple antibody binding. Taken together, our results suggest that antibodies to a specific protective epitope or epitopes on protein B5R are able to prevent EEV infection. The protein encoded by the B5R gene is therefore likely to play a crucial role in the initial steps of vaccinia virus infection—binding to a host cell and entry into its cytoplasm. © 1999 Academic Press

### INTRODUCTION

Vaccinia virus (VV) is a large complex DNA virus of the Poxviridae family. It replicates in the cytoplasm, and its genome encodes ~200 proteins (Goebel *et al.*, 1990). Two major infectious forms of VV have been described: intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Appleyard *et al.*, 1971; Ichihashi *et al.*, 1971; Payne, 1980). IMV is the major form that remains in the cytoplasm. It is composed of two tightly opposed membranes (Sodeik *et al.*, 1993) but can acquire two additional membranes derived from the *trans*-Golgi cisternae (Hiller and Weber, 1985; Schmelz *et al.*, 1994; Tooze *et al.*, 1993). The resulting intracellular enveloped virus (IEV) is transported by actin-containing microfilaments to the periphery of the cell (Cudmore *et al.*, 1995; Hiller *et al.*, 1981; Stokes 1976), where their outermost membrane fuses with the plasma membrane. These particles may remain attached to the cell surface as cell-associated enveloped virus (CEV) or may be released as EEV (Blasco and Moss, 1992; Ichihashi *et al.*, 1971; Morgan, 1976; Payne, 1979; Schmelz *et al.*, 1994). Even if EEVs represent a minor fraction of infectious particles, it is the biologically relevant form in terms of long-range dissemination of virus *in vitro* and *in vivo* (Payne, 1980). It has been well documented that immune responses

against EEV, and not against IMV, confer protection against orthopoxvirus infection (Appleyard *et al.*, 1971; Boulter, 1969; Boulter and Appleyard, 1973; Boulter *et al.*, 1971; Morgan, 1976; Payne, 1980; Payne and Kristensson, 1985; Turner and Squires, 1971).

Six genes are known to encode proteins present in the EEV outer envelope: A33R, encoding 23K–28K glycoproteins gp23-28 (Roper *et al.*, 1996); A34R, encoding gp22-24 (Duncan and Smith, 1992); A36R, encoding p45-50 (Parkinson and Smith, 1994); A56R encoding the hemagglutinin gp86 (Payne and Norrby, 1976; Shida, 1986); B5R, encoding gp42 (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992); and F13L, encoding a nonglycosylated 37K protein (Blasco and Moss, 1991; Hirt *et al.*, 1986). Except for the hemagglutinin, interference with the expression of any of these proteins by deletion or repression was shown to have a dramatic impact on the ability of the virus to spread from cell to cell in tissue culture and to form plaques (Blasco and Moss, 1991; Duncan and Smith, 1992; Engelstad and Smith, 1993; Herrera *et al.*, 1998; Mathew *et al.*, 1998; McIntosh and Smith, 1996; Roper *et al.*, 1988; Seki *et al.*, 1990; Wolffe *et al.*, 1993). However, modifications of these proteins seemed to have different consequences on EEV production and virulence (Smith, 1993). For example, the small plaque phenotype observed with F13L and B5R deletion mutants was probably due to a drastic decrease in EEV formation (Blasco and Moss, 1991; Engelstad and Smith, 1993; Wolffe *et al.*, 1993). However, deletion of the A34R or the A33R genes also resulted in a small plaque phenotype but in an increase in EEV formation (McIntosh and Smith,

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1996; Roper *et al.*, 1998; Wolffe *et al.*, 1997). Deletion of the hemagglutinin gene does not affect plaque size but rather causes the formation of syncytia (Ichihashi *et al.*, 1971; Shida and Dales, 1982). Modification of the genes were also shown to attenuate the virus *in vivo* (Blasco and Moss, 1991; Engelstad and Smith, 1993; McIntosh and Smith, 1996; Parkinson and Smith, 1994; Wolffe *et al.*, 1993). Even if these experiments demonstrated the importance of EEV-specific proteins, still very little is known about their involvement in EEV formation and egress from the infected cell, as well as their roles in attachment to the cell surface, entry, and uncoating.

In 1996, Ichihashi (1996) suggested that EEV entry involved binding to the host cell followed by endocytosis. Disruption of the EEV outer membrane would then allow the IMV form to fuse with the endosomal membrane and to release the viral core into the cytoplasm (Ichihashi, 1996). This hypothesis has recently been supported by the work of Vanderplasschen *et al.* (1998). The model implies that antibodies recognizing a specific cell attachment protein or proteins on the EEV should be able to interfere with the first step in infection, that is, binding to the host cell. Surprisingly, all attempts to identify antibodies that inhibit infection with EEV had failed so far (Ichihashi, 1996; Vanderplasschen *et al.*, 1997).

In contrast to these previous findings, we produced an antiserum against EEV envelope proteins ( $\alpha$ -VV-env.), which was able to neutralize EEV *in vitro*. Based on this result, we wanted to see whether it was possible to inhibit EEV infection with antibodies against individual EEV proteins. Disruption or modification of the hemagglutinin did not affect EEV production and infectivity (Galmiche *et al.*, 1997; Ichihashi and Dales, 1971), and the F13L gene product p37 is not exposed on the EEV surface but lines the inner side of the envelope (Schmutz *et al.*, 1995). We therefore tested the four remaining envelope proteins encoded by the genes A33R, A34R, A36R, and B5R for their ability to induce antibodies neutralizing EEV *in vitro* and to confer protection against a vaccinia virus challenge *in vivo*.

## RESULTS

*Anti-EEV envelope protein antibodies are able to neutralize EEV infection in vitro.* EEV is an IMV particle with an envelope containing a specific set of proteins. A detergent extract of this additional envelope was used to prepare the rabbit hyperimmune serum  $\alpha$ -VV-env., which we tested for its EEV neutralizing capacity. This antiserum recognized not only specific EEV proteins but also IMV components, which most probably contaminated the crude envelope preparation used for immunization (data not shown). Therefore, to exclude the possibility that the antiserum predominantly neutralized IMV or damaged EEV in the EEV preparation, the monoclonal antibody 5B4/2F2 against the 14-kDa IMV protein (Czerny and

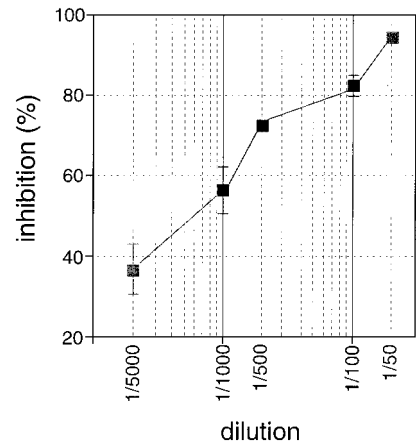
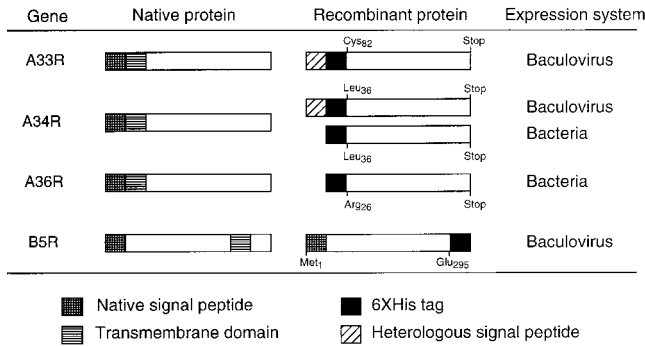


FIG. 1. *In vitro* neutralization of EEV by  $\alpha$ -VV-env. antiserum. The reduction of plaque numbers was calculated relative to control experiments using preimmune serum. Each value represents the mean  $\pm$  S.E.M. of duplicate measurements from at least four independent experiments.

Mahnel, 1990) was included in all *in vitro* neutralization experiments (Ichihashi, 1996; Vanderplasschen *et al.*, 1997). Preimmune serum was used as a negative control and as a standard to calculate relative inhibition.

Under these conditions,  $\alpha$ -VV-env. antiserum was able to neutralize freshly prepared EEV in a concentration-dependent manner (Fig. 1). At a dilution of 1:50, the antiserum neutralized 94.5% of the EEV; ~60% inhibition was observed when the serum was used at 1:1000. The observation that EEV can be neutralized by antibodies stands in sharp contrast to previous reports (Ichihashi, 1996; Vanderplasschen *et al.*, 1997).

*Production of antisera to individual EEV envelope proteins.* Because we had shown that it was possible to inhibit EEV infection with an antiserum against a mixture of all envelope proteins, we decided to prepare monospecific antisera against individual envelope proteins that might play a role in host cell attachment. We therefore expressed the extraviral portions of four likely candidates, the proteins encoded by the genes A33R, A34R, A36R, and B5R, as recombinant proteins in heterologous expression systems. Each of the recombinant proteins contained a terminal 6xHis tag for purification by affinity chromatography. These tags were added to the opposite ends with respect to the extremity naturally protruding out of the EEV particle, not to interfere with their native structure possibly involved in binding to a receptor or receptors on host cells. Furthermore, to govern secretion of the recombinant proteins originating from type II transmembrane proteins, a sequence encoding a heterologous signal peptide was added upstream of the truncated genes (Fig. 2). These strategies allowed recovery of the extraviral portions of the proteins A33R, A34R, and B5R directly from the supernatant of insect cells infected with recombinant baculovirus. However, the expression level of protein A34R was fairly low, and protein A36R



**FIG. 2.** Production of extraviral portions of EEV envelope proteins. Note that proteins A33R, A34R, and A36R are type II transmembrane proteins (see text for details). Amino acid numbering is according to the corresponding open reading frames in strain WR, and A33R, A34R, and A36R correspond to SalF3R, SalF4R, and SalF6R, respectively.

was not expressed at all using the baculovirus system. These two proteins were therefore expressed in bacteria, solubilized under denaturing conditions, and renatured during purification (see Materials and Methods).

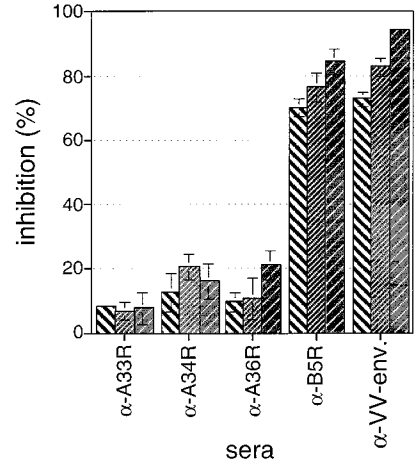
The different preparations of native EEV envelope proteins were used to immunize rabbits, and the specificities of the antisera were confirmed by immunoblotting (data not shown). Antibody titers were determined by ELISA on entire EEV particles (Table 1).

*In vitro* EEV neutralization by antisera to individual envelope proteins. The plaque inhibition assay previously performed with the  $\alpha$ -VV-env. antiserum was repeated using the antisera against individual envelope proteins at three different concentrations (Fig. 3). The anti-B5R protein antiserum neutralized EEV infectivity to nearly the same extent as the  $\alpha$ -VV-env. antiserum or even slightly higher at low concentrations: At a dilution of 1:5000, this serum showed 43% inhibition, whereas  $\alpha$ -VV-env. neutralized EEV to 37% (data not shown). For the other antisera, only marginal inhibition levels of up to 20% were observed. This might be due to a sterical hindrance to host cell attachment after antibody coating of the EEV surface. This hypothesis is in agreement with the observation that in these cases, the inhibition levels were less dependent on antibody concentrations. Inter-

**TABLE 1**  
Rabbit Hyperimmune Sera

Serum	Titer
$\alpha$ -A33R	1:2500
$\alpha$ -A34R	1:300
$\alpha$ -A36R	1:80
$\alpha$ -B5R	1:5000
$\alpha$ -VV-env.	1:10,000

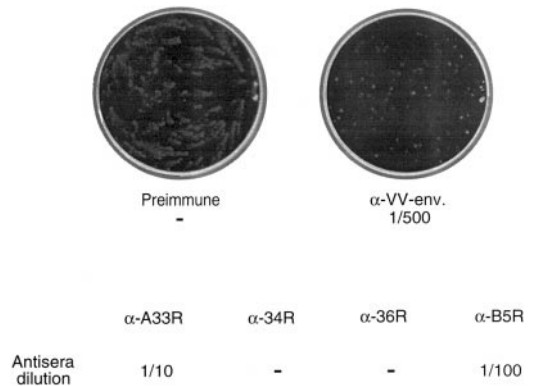
*Note.* The titers were determined by ELISA and represent the serum dilution resulting in the double of the OD<sub>490nm</sub> value obtained with preimmune serum.



**FIG. 3.** *In vitro* neutralization of EEV by antisera to individual EEV envelope proteins. Each serum was used at a dilution of 1:500, 1:100, and 1:50, shown in the left, middle, and right columns, respectively, of each group. The reduction in plaque numbers was calculated relative to the control with preimmune serum, and  $\alpha$ -VV-env. antiserum was used as a positive control for inhibition. Each value represents the mean  $\pm$  S.E.M. of duplicate measurements from at least four independent experiments.

estingly, the neutralizing activity of the anti-B5R protein was slightly increased in combination with either of the three other antisera.

The same antisera that neutralized EEV infectivity in the plaque inhibition assay, that is,  $\alpha$ -VV-env. and  $\alpha$ -B5R, also inhibited comet formation (Fig. 4). The size of the comets decreased with increasing antibody concentrations, and complete inhibition was obtained at dilutions of 1:500 and 1:100 for  $\alpha$ -VV-env. and  $\alpha$ -B5R, respectively. The anti-A33R protein antiserum also slightly inhibited comet formation at a dilution of 1:10, whereas  $\alpha$ -A34R and  $\alpha$ -A36R did not show any effect even at such high concentrations. It has been shown that inhibition of comet formation does not exclusively have to be due to neutralization of the freshly released EEV but that it may



**FIG. 4.** Inhibition of comet formation by monospecific antisera. The indicated dilution corresponds to the highest dilution that resulted in comet inhibition. —, No inhibition of comet formation.

TABLE 2

Amounts of Envelope Proteins in EEV

Protein	( $\mu\text{g}/\text{mg}$ ) EEV
A33R	<5
A34R	40
A36R	<10
B5R	30

be mediated via an inhibition of EEV release by an agglutination of the EEV on the cell surface (Vanderplasschen *et al.*, 1997). Nevertheless, it is interesting to note that some antibodies did and others did not inhibit comet formation, and that this characteristic correlated with EEV neutralizing activity in plaque inhibition tests.

*Relative amounts of envelope proteins in EEV.* The neutralizing activity of an antiserum might simply depend on the relative abundance of the corresponding protein on the EEV surface. We therefore estimated the relative amounts of the four envelope proteins in total EEV lysate through Western blotting using known amounts of the respective recombinant proteins as standards (Table 2). B5R and A34R represent the most abundant envelope proteins, whereas the amounts of A33R and A36R proteins were found to be almost an order of magnitude lower. Because the antiserum against protein A34R did not demonstrate any neutralizing activity, a nonspecific inhibition of EEV infectivity by opsonization with the  $\alpha$ -B5R antiserum seemed rather unlikely.

*In vivo protection of mice by vaccination with EEV envelope proteins.* To test whether the neutralizing activity of specific antisera *in vitro* correlated with protectivity *in vivo*, we vaccinated mice with the same recombinant proteins. After challenge with lethal doses of vaccinia virus, 90% of the mice vaccinated with B5R protein were protected. Surprisingly, vaccination with A33R protein

also provided protection, resulting in a 100% survival rate and in a significantly lower body weight loss and a more rapid recovery from infection than with B5R protein (Fig. 5).

Before challenge, a serum sample had been taken from each mouse to verify the antibody response to vaccination by ELISA and Western blotting (data not shown). Furthermore, in the case of vaccination with the A33R and B5R proteins, the ELISA serum titers were compared with the progression of infection after challenge (Fig. 6). For mice vaccinated with B5R protein, the quality of protection clearly paralleled the antibody titers observed before challenge. Mice with higher antibody titers recovered faster from infection, whereas the mouse showing the lowest antibody titer did not survive. Interestingly, no such association was found in mice vaccinated with A33R protein. Because the anti-A33R antiserum did not neutralize EEV *in vitro*, it was possible that the protection mediated by A33R protein was not due to a humoral but rather to a cellular immune response.

*Passive protection of mice by transfer of hyperimmune sera to EEV envelope proteins.* To test whether the protectivity of the B5R and A33R proteins *in vivo* was mediated by antibodies, we injected rabbit and mouse hyperimmune sera into mice having been challenged with vaccinia virus a few hours earlier. A significant proportion of mice that received  $\alpha$ -B5R antisera survived the challenge (Fig. 7), confirming our findings from the *in vitro* neutralization and active vaccination experiments. Surprisingly, however, best passive protection was obtained with mouse  $\alpha$ -A33R antiserum, whereas rabbit  $\alpha$ -A33R had no protective effect. All the mouse sera were subsequently tested in *in vitro* plaque inhibition assays. As for the rabbit sera, only the mouse  $\alpha$ -B5R antiserum was able to inhibit EEV infection *in vitro* (data not shown).

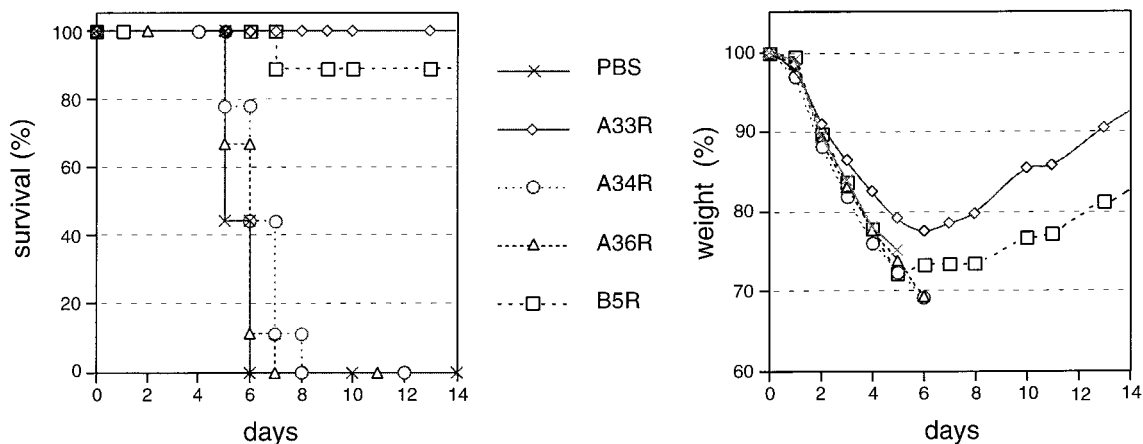
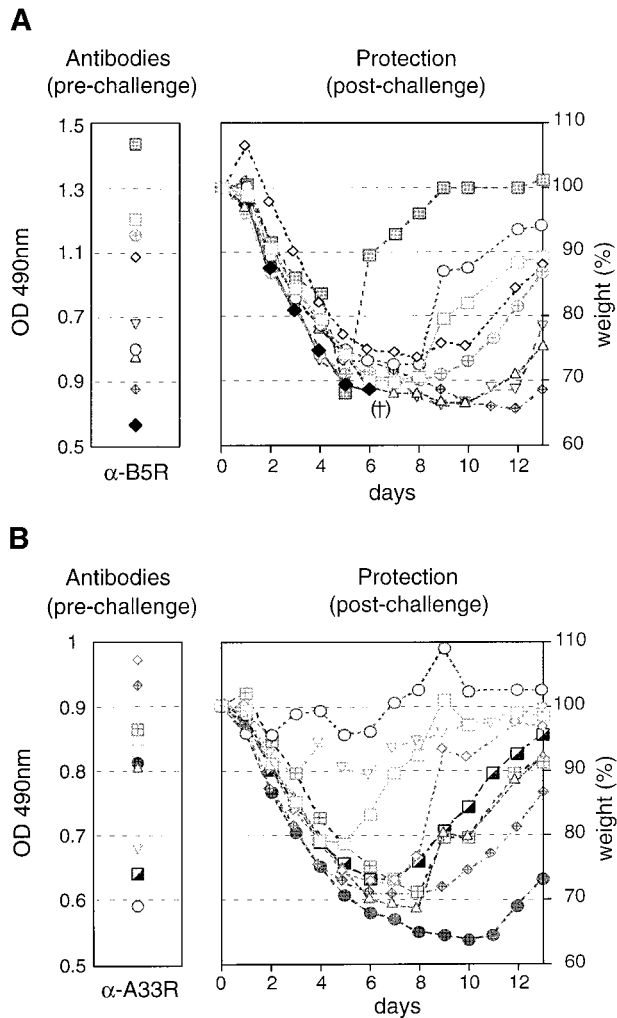


FIG. 5. Vaccination of mice with EEV envelope proteins. Survival rates and means of relative body weights are shown for groups of nine vaccinated mice after i.n. challenge with  $10^7$  pfu at day 0.





**FIG. 6.** Comparison of antibody titers and protectivity in individual vaccinated mice. Serum samples were taken from each mouse vaccinated with envelope proteins B5R (A) and A33R (B) before i.n. challenge with  $10^7$  pfu. The OD<sub>490nm</sub> values obtained by ELISA from the sera prechallenge and the relative body weights of the mice postchallenge are indicated.

*Protection of mice by DNA vaccination.* For technical reasons, two different expression systems had been used to produce the four recombinant proteins tested throughout this study. Because only mice immunized with baculovirus produced proteins had been protected in the vaccination studies, we had to verify that this result was not due to the different methods of protein preparation. DNA vaccination has been proved to offer a powerful alternative to classical vaccination protocols (Hassett and Whitton, 1996) and would allow us to compare the protective effects of the different EEV proteins in a more standardized manner. We therefore subcloned the full-length genes encoding the four envelope proteins into a eukaryotic expression vector. Except for the A33R construct, we were unable to detect expression of the genes in Western blots using lysates from transiently transfected cell lines (data not shown). Nevertheless, we

decided to vaccinate mice with these constructs and subsequently challenged them with lethal doses of vaccinia virus. Plasmids encoding the proteins A33R and B5R protected mice against infection (Fig. 8), thus confirming the results obtained with protein vaccination. Fifty percent of the mice immunized with A36R DNA survived after 2 weeks, but in contrast to those immunized with A33R and B5R DNA, they remained sick and did not regain their normal body weights even after 21 days (data not shown). In this case, cellular immunity might have played a role.

## DISCUSSION

The two infectious forms of vaccinia virions, IMV and EEV, differ in structure, antigenicity, and various other biological properties. They use different mechanisms to infect host cells, notably by binding to different cell surface receptors (Vanderplasschen and Smith, 1997). EEV represents the biologically relevant form of the virus to spread infection *in vitro* and *in vivo* (Blasco and Moss, 1992; Payne, 1980) and seems more selective in terms of affinity to different cells and tissues (Vanderplasschen and Smith, 1997). Recently, a novel model has been proposed suggesting that EEV entry consists of binding to the cell, endocytosis, and disruption of the EEV outer membrane within the acidic environment of the endosome, releasing IMV that gains access to the cytoplasm via the IMV infection mechanism (Ichihashi, 1996; Vanderplasschen *et al.*, 1998). Surprisingly, although IMV infectivity was readily inhibited by antibodies *in vitro*, two groups independently reported unsuccessful attempts to neutralize EEV using monoclonal antibodies and sera from infected animals and vaccinated humans (Ichihashi, 1996; Vanderplasschen *et al.*, 1997). Nevertheless, antibodies were shown to inhibit EEV release from infected cells, which explains why these antibodies were able to prevent comet formation (Engelstad *et al.*, 1992; Vanderplasschen *et al.*, 1997). This inhibition of virus egress might also represent one possible mechanism of antibody-mediated immunoprotection against vaccinia virus infection *in vivo*.

In this study, we produced antisera that not only blocked comet formation but also neutralized EEV infectivity in plaque inhibition assays. A rabbit hyperimmune serum against total EEV envelope lysate neutralized >94% of fresh EEV. An antiserum against recombinant protein B5R showed a similar effect, whereas antisera against three other EEV envelope proteins, A33R, A34R, and A36R, did not show any significant inhibition. The B5R gene product seems therefore to be a major target for antibody mediated inhibition of EEV infectivity. It is not surprising that the monoclonal antibodies used in the former reports did not show any neutralizing effect if they were not directed against the B5R protein or against the specific protective epitope on that protein. However, be-

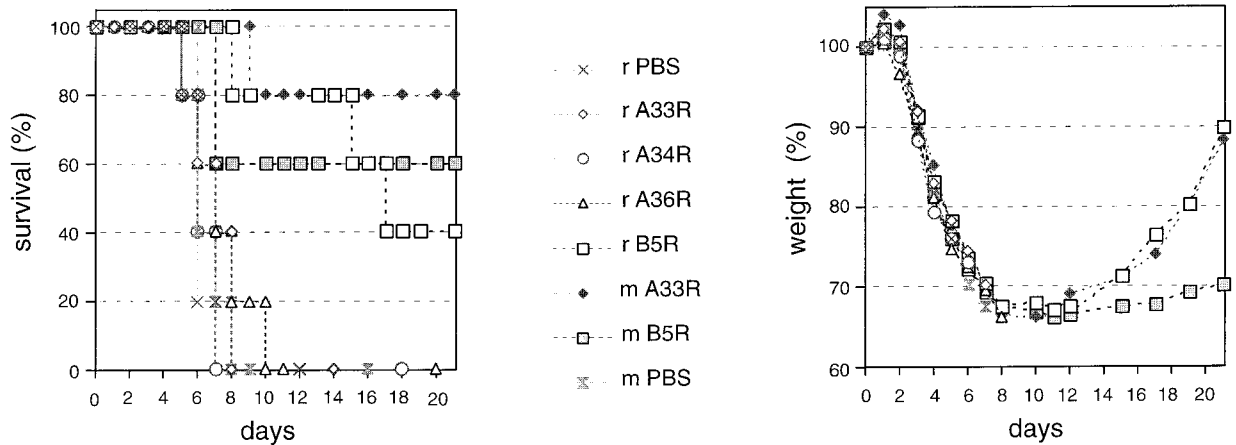


FIG. 7. Passive vaccination of mice with anti-envelope protein antisera. Groups of five mice were i.n. challenged with  $10^5$  pfu and received  $200 \mu\text{l}$  of rabbit or mouse antiserum 5 h later. Survival rates and mean values of relative body weights are shown. The r indicates rabbit antisera (open symbols), and the m indicates mouse antisera (closed symbols).

cause B5R is an abundant component of the EEV envelope and elicited high antibody titers after immunization, it is not clear why the polyclonal antisera used by the others did not inhibit plaque formation by EEV. A possible explanation for this apparent discrepancy might be that somehow the protective epitope on the B5R protein is not immunogenic during a natural infection or during vaccination using whole virions. This might represent a mechanism vaccinia virus has adopted during evolution to escape a first line of defense by the host's immune system. In contrast to the other groups, we might have bypassed this mechanism by presenting to the immune system an enriched fraction of protective B5R epitopes in the form of solubilized EEV membranes or of purified recombinant protein. In both cases, the lower abundance or the lack of otherwise immunodominant IMV proteins might be crucial.

Vaccination with recombinant extravirol domain of the B5R protein or with expression plasmids encoding the

full-length B5R gene protected mice against a lethal vaccinia virus challenge. This protectivity was most likely mediated by antibodies because mice showing higher anti-B5R antibody titers developed less severe infection and because nonvaccinated mice were passively protected by injection of anti-B5R hyperimmune sera. The A34R and A36R proteins did not show any protectivity. However, due to the poor expression levels of the A34R and A36R genes using the baculovirus expression system, these proteins had been expressed in bacteria and subsequently renatured for immunization. We therefore cannot exclude the possibility that authentic A34R and A36R proteins containing the same eukaryotic posttranslational modifications as their natural counterparts in the viral envelope might confer protection.

Unexpectedly, vaccination with the A33R gene product also provided a very efficient immunoprotection that could be passively transferred to naive animals. However, this protectivity did not correspond to specific an-

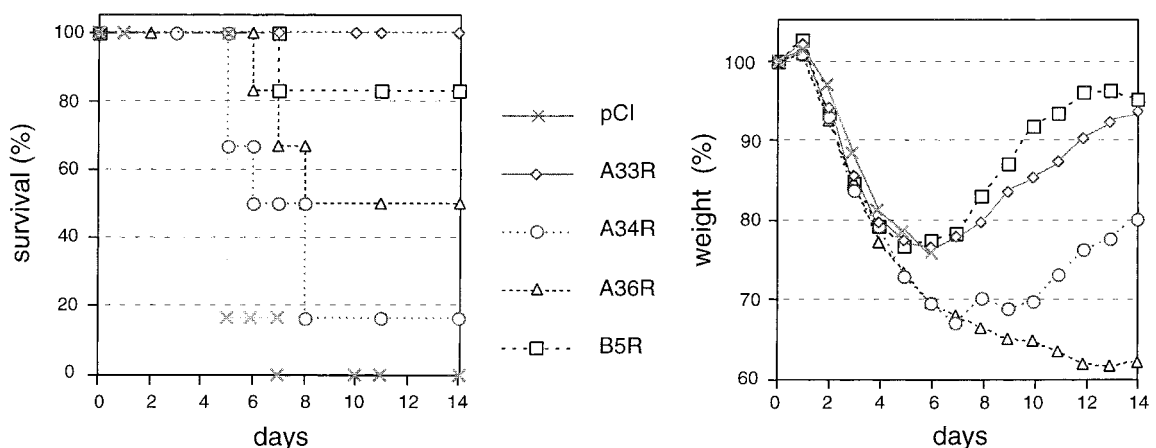


FIG. 8. Vaccination of mice with DNA encoding EEV envelope proteins. Survival rates and mean values of relative body weights are shown for groups of six vaccinated mice after i.n. challenge with  $10^6$  pfu.

tibody titers, and passive protection was obtained only with mouse and not with rabbit hyperimmune sera. Together with the finding that anti-A33R antibodies were unable to neutralize EEV *in vitro*, these results suggest that those antibodies do not directly interfere with the infection mechanism. Instead, it is possible that anti-A33R antibodies disrupt the virus dissemination pathway in conjunction with some homologous serum component like complement by destroying EEV particles or virus-infected cells. Alternatively, passive transfer of homologous effectors of cell-mediated immunity might have facilitated to build up an efficient cellular response, whereas their heterologous analogues in the rabbit serum did not. The protective mechanism may also involve Fc receptors on accessory cells (NK, macrophages), such that rabbit antibodies are less effective in mice. In any case, of the four envelope proteins tested, protein B5R remains the most likely target for direct antibody inhibition of infection and thus might play an important role in host cell attachment and/or penetration.

Knock-out mutants lacking the B5R gene showed a small plaque phenotype and produced a significantly smaller number of EEV due to a failure to wrap IMV particles, resulting in a greatly attenuated infectivity *in vivo* (Engelstad and Smith, 1993; Takahashi-Nishimaki *et al.*, 1991; Wolffe *et al.*, 1993). The extraviral N-terminal portion of the B5R protein contains four short consensus repeats (SCRs) with homologies to factor H, a component of the complement cascade (Engelstad and Smith, 1993; Takahashi-Nishimaki *et al.*, 1991). If B5R is involved in host cell attachment, as may be concluded from the present study, one would expect that deletion of portions of the corresponding extraviral domain would have a strong effect on EEV infectivity. Interestingly, deletion of one to three SCRs resulted in a large increase in the number of released EEV that produced small plaques (Mathew *et al.*, 1998), and mutants lacking all four SCRs produced normal plaques (Herrera *et al.*, 1998). All these deletion mutants apparently showed normal EEV infectivities *in vitro*. At first sight, this observation is difficult to reconcile with our results. One possible explanation might be that the short spacer region between SCR 4 and the transmembrane domain, which had not been deleted in those constructs, contains the epitope constituting the target for our neutralizing antibodies. In this case, the SCRs might provide a natural protection mechanism for the virus by shielding this sensitive epitope from recognition by cells of the immune system. This would provide a further possible explanation of why our antigen preparations, but not natural infection or vaccination with whole virus, stimulated the formation of protective antibodies. On the other hand, this hidden epitope would then be unlikely to play a role in cell attachment but might be involved in later events, maybe during penetration into a host cell. Alternatively, it still cannot be excluded that the anti-B5R antibodies neutralize EEV by

simply masking another, maybe yet unknown, envelope component involved in host cell attachment. Further studies to investigate the role of the B5R envelope protein in the initial steps of vaccinia virus infection are underway.

## MATERIALS AND METHODS

*Cells and viruses.* The rabbit kidney cell line RK13 (ATCC 37-CCL), the human osteosarcoma cell line 143B tk<sup>-</sup> (ATCC 8303-CRL), and the mouse fibroblastic cells NIH 3T3 (ATCC 1658-CRL) were cultured in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% heat-inactivated (30 min at 56°C) fetal calf serum (FCS) and 100 U/ml and 100 µg/ml penicillin and streptomycin, respectively. *Spodoptera frugiperda* (Sf9) insect cells (ECACC 89070101) were cultured at 28°C in SF-900 II medium (Life Technologies, Inc.) in the absence of serum as suspension culture in Erlenmeyer flasks shaken at 125 rpm or as monolayer.

Vaccinia virus strain IHD-J was used in this study, and EEV was prepared as described previously (Vanderplasschen *et al.*, 1997). Briefly, RK13 cells were infected with 1 plaque forming unit (pfu)/cell, and the supernatant was recovered 24 h postinfection. After clarification by low-speed centrifugation, the EEV suspension was diluted in DMEM containing 2% FCS, and any contaminating IMV infectivity was neutralized by the addition of monoclonal antibody 5B4/2F2 (Czerny and Mahnel, 1990) at a dilution of 1:1000.

*Production of the extraviral domains of EEV envelope proteins.* The portions of the genes A33R, A34R, A36R, and B5R encoding the extraviral domains of the corresponding proteins were amplified by PCR from genomic DNA of strain IHD-J. The primers used were based on the sequences in strain WR (Smith *et al.*, 1991) and are presented in Table 3. The resulting fragments were subcloned into pBluescript (pBS; Stratagene) via *Pst*I and *Hind*III sites contained within the primers, and the constructs were sequenced. To govern secretion of the type II proteins encoding by A33R, A34R, and A36R, a heterologous signal sequence originating from the human secretory component (Eiffert *et al.*, 1991; Krajci *et al.*, 1991; Rindisbacher *et al.*, 1995) was amplified by PCR and fused to the 5' ends of the fragments via *Sac*I and *Pst*I sites contained within the primers, according to the strategy shown in Fig. 2.

For purification by Ni<sup>2+</sup>-chelate affinity chromatography, all recombinant proteins were designed to contain a terminal 6xHis tag. We wanted to keep the terminal domains intact corresponding to the protruding extremities of the envelope protein *in vivo*. Therefore, for the type I protein encoded by the truncated B5R gene, the 6xHis tag was added to the C-terminus. For the type II recombinant proteins, the 6xHis tag was fused to the N-terminus, 1 amino acid downstream of the cleavage

TABLE 3  
Primers Used for PCR Amplification of Envelope Protein Encoding Genes

A33R	
Protein vaccination	+ 5'-GCCCTGCAGTGCATGTCTGCTAACGAG-3'
DNA vaccination	+ 5'-GAATCTAAGATATTATCATGATGACACCAGAA-3'
Protein + DNA vacc.	- 5'-GACAAGCTTAGTTCATTGTTTTAACACAAA-3'
A34R	
Protein vaccination	+ 5'-GCCCTGCAGCTGCATTACAAAGAAGAACTG-3'
DNA vaccination	+ 5'-CGAGAATTCCATTAATAAATGAAATCGCTTAAT-3'
Protein + DNA vacc.	- 5'-GACAAGCTTACTTGTAGAATTTTTAACACATA-3'
A36R	
Protein vaccination	+ 5'-GCCCTGCAGAGGAAAAAGATACGTAAGTCTGTC-3'
DNA vaccination	+ 5'-CGATCTAGATCAGAAATGATGCTGGTACC-3'
Protein + DNA vacc.	- 3'-GACAAGCTTACACCAATGATACGACCGA-3'
B5R	
Protein vaccination	+ 5'-GCCCTGCAGATGAAAACGATTTCCGTTGTTA-3'
	- 5'-GACAAGCTTA(ATG) <sub>6</sub> TTCTAACGATTCTATTTCTTGT-3'
DNA vaccination	+ 5'-CGACTCGAGTAAAAATGAAAACGATTTCCGTT-3'
	- 5'-CACGGTACCTTACGGTAGCAATTTATGGA-3'

Note. Sequences corresponding to authentic vaccinia virus protein coding regions are indicated in bold. The codons complementary to the 6xHis stretch are shown in italics.

site of the heterologous signal sequence, as will be described elsewhere (Galmiche *et al.*, unpublished observations). The resulting sequences were subcloned into pBlueBac4 (Invitrogen) to generate recombinant baculoviruses for the expression of native proteins. Alternatively, similar constructs without signal sequences were inserted into pQE vectors (Qiagen) and expressed in bacteria. The bacterial products were bound to Ni<sup>2+</sup> columns under denaturing conditions, renatured stepwise with PBS, and eluted under native conditions. Briefly, total bacterial lysates were solubilized with 6 M guanidine hydrochloride and loaded onto Ni<sup>2+</sup>-NTA-agarose beads (Qiagen), according to the manufacturer's recommendations. The columns were washed with a linear gradient of 8–0 M urea in 0.1 M phosphate/0.01 M Tris buffer, pH 8. Bound renatured proteins then were eluted with the same buffer containing 200 mM imidazole.

**Antibodies.** Table 1 describes the antisera against vaccinia virus proteins used in this study. The rabbit  $\alpha$ -VV-env. was obtained from an animal that had been immunized subcutaneously four times at 4-week intervals with total EEV membrane protein prepared as described previously (Payne, 1979). To generate antisera against the recombinant envelope proteins, 100–200  $\mu$ g of each native protein was injected five times at 4-week intervals. The B5R and A33R gene products were obtained in sufficient amounts from the baculovirus system. However, The A36R protein was produced in bacteria and renatured as described above, and for the A34R protein, a mixture of baculovirus and bacteria protein was injected. All antisera were produced using Freund's complete and incomplete adjuvant for primary and boost injections, respectively. The antibodies were stored at –20°C and heated for 30 min at 56°C before use to

inactivate complement. The titers of the sera were determined by ELISA on EEV (see below).

**Antibody titration.** Nunc 96-well plates (Immunoplate MaxiSorp) were coated with wildtype EEV (1  $\mu$ g virus/well) in PBS at 4°C overnight. After three washes with PBS, the virus was fixed with 2% formaldehyde for 30 min at 4°C. After one wash, nonspecific binding sites were blocked with 5% nonfat dry milk in PBS for 1 h at room temperature. Duplicate samples of antibody dilution series were then added to the fixed virus and incubated for 1 h at room temperature. After washing, anti-rabbit IgG antibody conjugated with horseradish peroxidase was added at a dilution of 1:1000. Using 1,2-phenylene-diamine as a substrate, bound antibody was quantified in an ELISA reader at 490 nm. Each experiment was performed at least three times. The titer of each antiserum was defined as the dilution resulting in a signal corresponding to the double of the value obtained with preimmune serum.

**Antibody neutralization assay.** Fresh EEV (150–200 pfu/ml) in DMEM containing 2% FCS were incubated for 1 h at 37°C with the specific antisera at variable concentration or with preimmune serum as a negative control. In addition, to neutralize any contaminating IMV or damaged EEV, the monoclonal antibody 5B4/2F2 was added (Vanderplasschen *et al.*, 1997) at a dilution of 1:1000. Of these mixtures, 500  $\mu$ l/well was then directly added to confluent 143B tk<sup>-</sup> cells in six-well plates and incubated for 1 h at room temperature. After two washes with PBS, the cells were incubated for 2 days at 37°C under liquid overlay, and plaques were counted after staining with 0.1% crystal violet.

**Comet inhibition assay.** RK13 cells grown in six-well dishes were infected with IMV (50–100 pfu/well) for 1 h at room temperature. After washing, the cells were overlaid



with 1 ml of DMEM containing 2% FCS and varying concentrations of specific antisera or preimmune serum as a control. The cells were incubated for 2 days at 37°C and then stained with crystal violet.

**Immunoblotting.** Total EEV proteins and purified recombinant EEV envelope proteins were fractionated on 10% or 15% SDS-polyacrylamide gels and subjected to immunoblotting. The membranes were incubated with rabbit anti-A33R, anti-A34R, anti-A36R, or anti-B5R antiserum at a 1:1000 dilution in TBS-Tween for 1 h at room temperature. Bound antibody was detected using an anti-rabbit IgG antibody coupled to horseradish peroxidase and the enhanced chemiluminescence kit from Amersham. EEV envelope proteins were quantified by densitometric scanning of individual signals (Elscrip 400; ATH Hirschmann, Germany) and by comparison with the densities of the corresponding bands obtained with known quantities of the purified proteins.

**Protection of mice by vaccination with individual envelope proteins.** Six-week-old female BALB/c mice were immunized subcutaneously four times at 2-week intervals with the different recombinant EEV membrane proteins (10 µg/injection) emulsified in Freund's complete and incomplete adjuvant for primary and boost injections, respectively. The mice were then challenged intranasally (i.n.) with 10<sup>7</sup> pfu in 20 µl of PBS, with the animals under deep anesthesia. Their body weights were monitored daily, and animals that had lost >30% were killed. The amount of specific antibody contained in a blood sample taken before challenge was determined by ELISA (see above).

**Protection of mice by passive transfer of hyperimmune sera.** Six-week-old naive mice were anesthetized and infected i.n. with 10<sup>5</sup> pfu in 20 µl of PBS. Five hours after challenge, they received intraperitoneally (i.p.) 200 µl of mouse or rabbit hyperimmune serum against individual recombinant envelope proteins. Infection was monitored via the body weight of the animals as described above.

**Protection of mice by DNA vaccination.** The complete open reading frames A33R, A34R, A36R, and B5R from strain IHD-J were amplified by PCR using primers containing unique restriction sites (Table 3) and inserted into the eukaryotic expression vector pCI (Promega). Six-week-old female BALB/c mice were immunized by four intramuscular injections without adjuvant at 2-week intervals with these constructs or with empty vector as a control (100 µg in 100 µl of PBS/injection). The mice were then challenged i.n. with 10<sup>6</sup> pfu in 20 µl of PBS, with the animals under deep anesthesia. Infection was monitored via the body weight of the animals as described above.

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