Immune Response to Vaccinia Virus Recombinants Expressing Glycoproteins
gE, gB, gH, and gL of Varicella-Zoster Virus

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Immunogenicity of Varicella-zoster virus glycoproteins gE, gB, gH, and gL expressed by recombinant vaccinia viruses (VV) separately or simultaneously was determined in mice and guinea pigs by ELISA, Western blotting, radioimmunoprecipitation, plaque reduction assay, and skin test. Single VV-gE and VV-gB recombinants and double VV-gH/gL recombinant elicited specific antibodies with VZV neutralizing activity in mice. Co-expression of gE and gB by one recombinant VV resulted in an increased antibody response in comparison with immunization with single recombinants or their mixtures. Unlike anti-gB and anti-gH/gL antibodies, the gE-specific antibodies had no virus neutralizing activity in absence of complement, and when used alone, they even caused considerable increase of VZV infectious units. Moreover, immune sera containing anti-gE antibodies antagonized complement independent virus-neutralizing activity of anti-gB- and anti-gH/gL-positive sera. The ability to induce delayed hypersensitivity reaction to VZV antigens was observed after immunization of guinea pigs with gE- and/or gB-expressing VVs.

Key Words: varicella-zoster virus; herpesviruses; glycoproteins; vaccinia virus; vaccine; antibodies; cellular immunity; virus neutralization; immunization.

INTRODUCTION

Varicella-zoster virus (VZV) is responsible for chickenpox and shingles. The host reactivity to VZV involves both humoral and cell-mediated immune responses (for review, see Arvin, 1995). The major viral antigens of VZV are the glycoproteins (gps), gE, gB, gH, and gL, which are structural components of the virion envelope. Glycoprotein gE (M, 90–98 kDa) is the most abundant viral gp in VZV-infected cells (Montalvo and Grose, 1985). This glycoprotein is highly modified by both N- and O-linked sugars, sialiation, myristylation, palmtiylation and sulphation, as well as by serine/threonine and tyrosine phosphorylation (Grose, 1990; Harper and Kangro, 1990; Namazue et al., 1989). In infected cells, VZV gE forms a noncovalently linked complex with VZV gI (Yao et al., 1993). Owing to two targeting signals (Alconada et al., 1996; Zhu et al., 1996), gE is targeted to trans-Golgi network (TGN). The gE molecule binds Fc fragment of IgG. Usually, antibodies against gE are able to neutralize VZV infection in vitro in the presence of complement only (Grose and Litwin, 1988; Ludvíková et al., 1991); however, complement-independent neutralizing monoclonal gE antibodies have been also prepared (Wu and Forghani, 1997). The second most strongly immunogenic glycoprotein of VZV, gB, is a heterodimer of two polypeptide chains (M, 66 and 65 kDa) that are linked by disulphide bonds. Molecule of gB contains both N- and O-linked sugars, and it is sialiated, sulphated, and palmtiylated (Grose, 1990). The gB was found to bind to cell-surface heparan sulphate proteoglycans (Jacquet et al., 1998). Glycoprotein gB elicits complement-independent virus-neutralizing antibodies (Edson et al., 1985a,b). The gB-specific antibodies inhibit VZV-induced cell-to-cell fusion in infected monolayers (Montalvo and Grose, 1987). The third most abundant VZV glycoprotein, gH, is associated with another viral glycoprotein, gL, with chaperone function (Forghani et al., 1994), and this complex is transported to the cell surface (Dus et al., 1995). The gH:gL complex is responsible for viral entry, egress, cell fusion, and cell-to-cell spread of the VZV in cultured cells. Glycoprotein gH induces complement-independent neutralizing antibodies. Two other glycoproteins, gC and gL, induce low-level immune responses (Huang et al., 1992). Also some non-glycosylated viral proteins like IE62 or thymidine kinase are significant targets of host-immune responses. Although cell-mediated immune response is more important for limiting the spread of virus and clearing infection (Arvin, 1995), antibodies also substantially interfere with VZV replication in vivo as is evident from reduction of severity of varicella after administration of high-titer VZV immunoglobulin (Zaia et al., 1983). The exact role of particular viral antigens in induction of immune status is not well understood. It has been shown that antibody responses to gE and gB after natural infection and after vaccination with OKA vaccine is highly variable from one individual to another; however, these
two glycoproteins are predominant among other viral antigens in respect to induction of VZV-neutralizing antibodies (Haumont et al., 1997).

We have recently shown that VZV glycoproteins gE and gH:gL expressed by recombinant vaccinia virus (Ludvíková et al., 1991; Němečková et al., 1996) elicit antibodies with virus-neutralizing activity in mice. The goals addressed in this paper were to better characterize the main immunogenic VZV glycoproteins that are supposed to be included into a subunit vaccine against VZV, to determine the contribution of the particular glycoproteins to humoral and cellular immune responses, and to evaluate the influence of coexpression of the glycoproteins. For this purpose, we prepared a set of single and double VV recombinants expressing four VZV genes (gE, gB, gH, and gL) and used them for immunization of mice and guinea pigs. Humoral and cell-mediated immune responses to VZV antigens were determined.

RESULTS

Antibody response of mice inoculated with gE- and gB-expressing recombinant VVs

To evaluate the immunogenicity of VV-VZV recombinants, the antibody responses of mice inoculated with various doses of different recombinants (Table 1) and of their mixtures were analyzed. Results of the examination of sera of mice after administration of VV-expressing gE and gB are shown in the upper part of Table 2. The results obtained by ELISA and WB indicate that both glycoproteins are relatively good immunogens in mice since the administration of $1 \times 10^6$ PFU (Groups 1 and 2) of either VV-gE or VV-gB induced specific antibodies. As observed for the VV-gB recombinant, the amount of antibodies formed depended on the dose of the recombinant virus administered but only within a certain range of doses (Groups 2–4). The data on gE and gB antibodies monitored by ELISA and WB were in good agreement. To test the immunogenicity, mixtures of the two recombinants containing one dose of VV-gE ($1 \times 10^6$ PFU) and increasing doses of VV-gB ($1 \times 10^6$, $3 \times 10^6$, or $10 \times 10^6$ PFU) were co-injected (Groups 5–7). To further determine the
effect of co-administered virus, VV-gE was replaced by the control VV-gL virus (Groups 9–11). VV-gL was chosen because it does not induce VZV-specific antibodies reactive in the tests used (Group 12). As evidenced by both ELISA and WB, the presence of increasing amount of the second VV in the mixture reduced the antibody response to the gE antigen. Inoculation of a mixture of VV-gE and VV-gB recombinants and in particular the double VV-gE/gB recombinant induced higher levels of antibodies against the complex VZV antigen than did any single VV recombinant tested. The VZV-neutralizing activity of sera from all groups immunized with gE- and/or gB-expressing VVs was measured using plaque reduction test. In agreement with previous reports, sera of mice immunized with VV-gE neutralized VZV only in the presence of complement (Group 1). On the other hand, gB-specific virus-neutralizing antibodies were detected both in the presence and absence of complement, and the magnitude of neutralization titers was in good agreement with the values found by the other tests used (Groups 2–4). The sera precipitated both a fully processed 118-kDa form of gH combined with a 19-kDa form of gL and a premature 97-kDa form of gH. If the mice were immunized with mixtures of both double recombinants VV-gH/gL and VV-gE/gB, we observed that increasing the dose of latter virus in the mixture resulted in decrease of the antibodies elicited by the former virus (Groups 8–10). This is an observation similar to that seen after immunization with the mixtures of VV-gE and gB (Table 2).

The virus-neutralizing capacities of sera of mice inoculated with the single VV-gL or VV-gH recombinant, with their mixtures, or with the double recombinant VV-gH/gL were also compared. Administration of single recombinants or their mixtures at a dose of $1 \times 10^7$ PFU did not elicit virus neutralizing antibodies of a titer $\geq 4$ (not shown). The presence of high titers of virus-neutralizing antibodies after immunization with $1 \times 10^6$ PFU of double recombinant VV-gH/gL (Table 4, Group 4) confirmed that the genes coding for gH and gL must be expressed simultaneously in the same cell to process the gH:gL immunogen properly. We compared the efficacy of the administration of both double recombinants VV-gE/gB and VV-gH/gL mixed at various ratios (Table 4). The simultaneous inoculation of both viruses (Groups 1–3) did not result in an increase of virus neutralizing antibody titers in comparison with the inoculation of one recombinant only (Groups 4–7). Raising the VV-gE/gB virus dose in mixtures (Groups 2 and 3) resulted in a reduction of complement-independent virus-neutralizing antibodies. A similar negative effect of anti-gE antibodies was seen also in Experiment 1 shown in Table 2 (Groups 5–7). This decrease of titers either could be the result of

### Antibody Response of Mice Immunized with VVs Expressing gH and/or gL as Detected by RIPA

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dose of virus inoculated i.p. into mice</th>
<th>Peak area of gp precipitated [OD]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>gH (118 kDa)</td>
</tr>
<tr>
<td>1</td>
<td>VV-gL $1 \times 10^7$</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>VV-gH $1 \times 10^7$</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>VV-gH $1 \times 10^7$ + VV-gL $1 \times 10^7$</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>VV-gH/gL $1 \times 10^7$</td>
<td>8204</td>
</tr>
<tr>
<td>5</td>
<td>VV-gH/gL $1 \times 10^6$</td>
<td>7549</td>
</tr>
<tr>
<td>8</td>
<td>VV-gH/gL $1 \times 10^4$ + VV-gE/gB $1 \times 10^4$</td>
<td>4612</td>
</tr>
<tr>
<td>9</td>
<td>VV-gH/gL $1 \times 10^4$ + VV-gE/gB $3 \times 10^4$</td>
<td>2627</td>
</tr>
<tr>
<td>10</td>
<td>VV-gH/gL $1 \times 10^4$ + VV-gE/gB $10 \times 10^4$</td>
<td>1514</td>
</tr>
</tbody>
</table>
Neutralization titers of sera

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dose of virus inoculated</th>
<th>Neutralization titer of sera (Complement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VV-gH/gL 1.10^{10}</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>VV-gH/gL 1.10^{6}</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>VV-gH/gL 1.10^{4}</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>VV-gH/gL 1.10^{6}</td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td>VV-gE/gB 1.10^{6}</td>
<td>&lt;4</td>
</tr>
<tr>
<td>6</td>
<td>VV-gE/gB 3.10^{6}</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>VV-gE/gB 10.10^{6}</td>
<td>16</td>
</tr>
</tbody>
</table>

a,b For explanations, see Table 2.

Cutaneous reaction to VZV antigens

Cellular immune responses of guinea pigs immunized with recombinant VV were tested by the skin test. Cutaneous reactions were measured 48 h after intradermal inoculation of test antigen. The results of two immunization experiments are shown in Table 5. In the first experiment, guinea pigs were infected with seven different recombinant VVs, a mixture of two double recombinants, extracellular VZV-Oka, or with PBS. The table shows mean cutaneous reaction values ± standard deviation (SD). As compared with reactions after VV-preS2S inoculation taken as a background negative control, weakly positive cutaneous reactions were induced in animals immunized with VV-gE or VV-gB. The differences from negative control were not statistically significant from what could be caused by great variability of animal responses. However, we observed several times that positive reactions to VZV antigen were accompanied by more intensive colouring of erythema (not shown). In animals immunized with VV-gE/gB or with a mixture of VV-gE/gB + VV-gH/gL, the mean values of skin reaction size differed from the control group significantly ($P = 0.029$ and $P = 0.009$, respectively). The response in animals immunized with VZV-Oka also showed a significant difference ($P = 0.021$). No positive cutaneous reaction was observed in controls injected with PBS only or in animals infected with the single recombinants VV-gH, VV-gL or, surprisingly, with the double recombinant VV-gH/gL.

Sera of all animals were tested for the presence of anti-VZV and anti-VV antibodies 5 weeks after immunization. Inoculation of recombinant VVs elicited anti-VV responses in all animals. All sera also contained VZV-neutralizing antibodies. Since the sera of groups inoculated with PBS and VV-preS2S displayed VZV-neutralizing activity at titers of 8–16, it is obvious that at least a portion of these antibodies was produced in response to the administration of skin-test antigens. A finding of relatively high neutralization activity in sera from animals immunized with VV-gE/gB, VV-gE, or VV-gB confirmed an immunizing capability of VV-expressed gE and gB in guinea pigs. Unlike mice, guinea pigs were apparently less capable of responding to VV-expressed gH/gL by production of VZV-neutralizing antibodies, and this might have been associated with their failure to develop skin reactivity. To investigate this point further and to confirm the most important data from Experiment I, another experiment (Table 5, Experiment II) was carried out in which VVgE/gB and VVgH/gL recombinants were used for immunization and the skin test was only performed in a portion of the animals. We found no cutaneous reaction and no antibodies reacting with VZV in neutralization test in VV-gH/gL-inoculated animals, which were not boosted by skin test antigen.
DISCUSSION

Immune response to particular glycoproteins following infection with VZV has been well documented (Weigle and Grose, 1984; Brunell et al., 1987; Dubey et al., 1988; Arvin et al., 1991; Haumont et al., 1997), but data on immunogenicity of separately expressed VZV glycoproteins are less numerous (Vafai and Yang, 1991; Vafai, 1993; Ludvíková et al., 1991; Lowry et al., 1992; Massaer et al., 1993; Němečková et al., 1996). The results of the present series of experiments demonstrated that VV recombinants expressing VZV gE, gB, or gH + gL glyco-

![Graph A](image1.png)

**FIG. 1.** Enhancement of VZV plaque formation by immune sera of mice inoculated with VV-gE. (A) Plaque reduction and plaque enhancement by gE-positive sera in the presence (■) and absence (□) of complement, respectively. The gE-positive sera were pooled from mice immunized with $10^5$ VV with the gE gene inserted into hemagglutinin of VV. The relative numbers of plaques in all three graphs represent percentages of plaque counts in samples without serum. (B) Inhibition of complement-independent virus neutralization activity of anti-gB-positive serum by anti-gE positive serum. One dilution (1:6) of anti-gE-positive serum (after immunization with VV-gE) was mixed with serial dilutions (1:8–1:512) of anti-gB positive serum. Virus neutralization activity was tested either in the presence ($C^+$) or absence ($C^-$) of complement. (C) Inhibition of complement-dependent virus neutralization activity of anti-gH/gL-positive serum by anti-gE-positive serum but not by other sera. Sera of mice immunized with 1-VV-gE, 2-VV-gB, 3-VV-gH, 4-VV-gL, 5-VV-preS2-S, or 6-VV(TK-) diluted 1:6 were mixed with serum of mice immunized with VV-gH/gL, diluted 1:16 or 1:32, or the sera were used unmixed (no gH/gL). The last two bars (7-none) represent virus neutralization by unmixed anti-gH/gL. The mixed sera and controls were assayed by the plaque reduction test in the absence of complement.
protein genes were able to induce gE, gB, and gH-specific antibodies, respectively, which displayed VZV-neutralizing activity. Immunization with single recombinants or with mixtures of single recombinants was, in all combinations, less efficient than immunization with double recombinants. It had already been known for some time that to induce anti-gH neutralizing antibodies, gH had to be expressed together with gL in the same cell; doses lower than 2.10^7 PFU of VV-gH and VV-gL administered intraperitoneally did not induce virus-neutralizing antibodies in mice (Némecková et al., 1996). In the present experiments, immunization with the VV-gE/gB double recombinant resulted in much higher titers of neutralization antibodies than those obtained with mixtures of the corresponding single recombinants. This might have been a consequence of temporary interaction between these two glycoproteins in the course of their synthesis; it should be remembered that a complex, heterooligomeric organization of glycoproteins has also been found in other herpes viruses (Handler et al., 1996). However, our efforts to co-precipitate the gE/gB complex 24 h after infection with VV-gE/gB and crosslink it with dithio-bis(succinimidylpropionate) (Marešová, results not shown) have not been successful so far. Immunization with mixtures of two double recombinants, VV-gE/gB and VV-gH/gL, did not increase neutralization titers in postinfection sera. Cellular immune responses to glycoproteins gE, gB, and gH have been found in subjects with acute and convalescent varicella (Arvin et al., 1986; Giller et al., 1989) as well as in guinea pigs inoculated with the Oka varicella vaccine (Sato et al., 1998). We observed that glycoproteins gE and gB expressed by VV were able to induce delayed-type hypersensitivity in guinea pigs similarly as did immunization with extracellular VZV-OKA. We could not find any significant skin reaction after immunization with VV-gH/gL or VV-gH plus VV-gL, although the presence of VV-specific antibodies in all guinea pigs indicated that all animals were infected with VV. Furthermore, the titers of anti-VV antibodies in animals inoculated with VV-gH/gL, VV-gB, or VV-gE/gB did not differ markedly. It could be speculated that the amount of gH/gL formed was sufficient to stimulate virus-neutralizing antibodies in mice but failed to elicit neutralizing antibodies and delayed-type skin reaction in guinea pigs. 

### Table 5

Cutaneous Reaction and Antibody Responses in Guinea Pigs Immunized with VV-VZV Recombinants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Area [mm²]</th>
<th>Antibodies [Av 442 nm ± SD]</th>
<th>Neutralization titer of sera C³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N³</td>
<td>VZVag</td>
<td>VVag</td>
</tr>
<tr>
<td>VV-preS2S</td>
<td>3</td>
<td>20.0 ± 24.9</td>
<td>0.196 ± 0.117</td>
</tr>
<tr>
<td>VV-gE/gB</td>
<td>3</td>
<td>57.7 ± 8.5</td>
<td>0.029³</td>
</tr>
<tr>
<td>VV-gH/gL</td>
<td>3</td>
<td>9.3 ± 19.0</td>
<td>0.110</td>
</tr>
<tr>
<td>VV-gE/gB + VV-gH/gL</td>
<td>4</td>
<td>76.3 ± 17.0</td>
<td>0.009*</td>
</tr>
<tr>
<td>VV-gE</td>
<td>3</td>
<td>100.7 ± 73.5</td>
<td>0.083</td>
</tr>
<tr>
<td>VV-gB</td>
<td>3</td>
<td>107.0 ± 112.9</td>
<td>0.183</td>
</tr>
<tr>
<td>VV-gH</td>
<td>3</td>
<td>−21.0 ± 36.3</td>
<td>0.113</td>
</tr>
<tr>
<td>VV-gL</td>
<td>3</td>
<td>48.3 ± 16.5</td>
<td>0.107</td>
</tr>
<tr>
<td>VZV/Oka</td>
<td>3</td>
<td>176.3 ± 98.0</td>
<td>0.021*</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>0 ± 0</td>
<td>0.296 ± 0.031</td>
</tr>
</tbody>
</table>

Note. Area values are means ± SD.

³ Number of animals in group.

¹ In Experiment I, 60 μg of VZV antigen was intradermally injected 18 days after administration of recombinant VV. Skin reaction area was measured 48 h after injection of antigen. Guinea pigs were bled out 5 weeks after inoculation of recombinant VV. VZV- and VV-specific antibodies were determined in individual sera. VZV-neutralizing activity was measured in pooled sera. Cutaneous reactions in animals inoculated with the various VV-VZV recombinants were compared with animals immunized with the VV-preS2S recombinant.

² Statistical significance was tested by the unpaired t test. * Indicates that the size of cutaneous reaction areas differed significantly from animals originally inoculated with VV-preS2S (in the 95% confidence interval).

³ The animals in Experiment II were injected on the same time schedule and with the same doses of VV and VZV antigen as in Experiment I. Significance of the differences between cutaneous reactions in VV-gE/gB- and VV-gH/gL-injected animals was tested.

₄ Skin test was not performed.
pigs. It is likewise possible that the processing of these two glycoproteins is less efficient in guinea pig than in murine cells.

We also observed that when anti-gE-positive sera were used in the absence of complement in neutralization assays, the number of plaques increased. Antibody-dependent enhancement (ADE) of virus infection in vitro was described for several viruses, including human immunodeficiency virus type 1 (Homsy et al., 1990), influenza virus (Ochiai et al., 1990), and respiratory syncytial virus (Gimenez et al., 1989). ADE in these viruses can be explained so that the exposed Fc portions of the antibodies that are bound to virus particles bind to Fc receptors on the surfaces of cells, thereby increasing the internalization of the virus. This explains the mechanism of ADE in macrophages or monocytes but is not likely for VZV infection of fibroblastoid LEP cells used in our study. We also established that ADE was not due to an enhanced stability of the gE-antibody-treated virus (results not shown).

At this writing, the nature of this observation is not clear; however, it might be associated with some other phenomena described recently. It has been observed by Shiraki et al. (1997) that postinfection treatment of cells with the gE-gI complex isolated by affinity chromatography inhibited VZV plaque formation. The effect was dose dependent and treatment of VZV-infected cells with 100 μg of purified glycoprotein reduced plaque number by 85%. VZV glycoproteins (Shiraki and Takahashi, 1982; Montalvo and Grose, 1986), including gE (Maresˇova´ et al., 2000), have been shown to be released from cells infected with VZV or recombinant VV-gE, and therefore the cell-free virus stocks prepared by sonication of VZV infected cells may contain such inhibitory gE-gI complexes. It follows that the actual titer of virus in different stocks may depend on the concentration of infectious virions and at the same time on the concentration of inhibiting gE-gI complexes. Thus, the role of anti-gE antibodies in ADE could be dependent on the binding of the inhibitory antigens present in the preparations of cell-free VZV used in the plaque reduction test. Formation of antigen–antibody complexes could prevent the inhibitory effect of free gE-gI on VZV infection and cause an increase in the plaque count in vitro. The differences in the content of free VZV glycoproteins in cell-free virus stocks used in different laboratories for neutralization tests might be responsible for different findings concerning the capability of anti-gE antibody to neutralize VZV without complement. The other mechanism operative may involve the nature of gE antibodies used. Polyclonal antibodies elicited by VV-gE recombinants may have a higher affinity to soluble gE-gI complexes than monoclonal antibodies used by other investigators.

Another possible mechanism of VZV infection enhancement by anti-gE could be based on the fact that gE is internalized from the membrane of infected cells by endocytosis (Olson and Grose, 1997). It has been observed that antibodies to gE of pseudorabies virus, another alphaherpes virus, induced endocytosis and caused bridging and capping of membrane glycoproteins on infected cells (Favoreel et al., 1999). It is possible that increased ligand-induced endocytosis could result in a higher rate of infection.

Our results suggested that all three glycoproteins, gE, gB, and gH/gL, would be important components for a future subunit VZV vaccine. Although neither gE nor gB required co-synthesis of other glycoproteins so essentially as gH required gL, the positive immunization effect of VV-gE/gB suggested that interaction of viral glycoproteins could occur during their synthesis or processing and that the simultaneous synthesis of recombinant glycoproteins in the same cell could be worth considering in designing a subunit vaccine.

MATERIALS AND METHODS

Viruses and cells

All recombinant vaccinia viruses (VV) were prepared from clone P13 generated from smallpox Sevac VARIE vaccine (strain Praha) (Kutinovav et al., 1995). A list of single and double recombinants containing VZV gene 37 (gH), gene 60 (gL), gene 31 (gB), or gene 68 (gE) inserted in the VV thymidine kinase (TK) or hemagglutinin (HA) genes is shown in Table 1. Expression of extrinsic genes was controlled by VV early/late p7.5k or late 11k promoters. Construction of single recombinant VVs expressing VZV gE, designated VV-gE (Ludvikovav et al., 1991) and expressing gH and gL and designated VV-gH and VV-gL, respectively (Nemeckovav et al., 1996), were described previously. Single recombinant VV-expressing gB and double recombinants VV-gE/gB and VV-gH/gL were prepared recently (Marešovav et al., 2000). Electrophoretic pattern of glycoproteins produced by recombinants used in the present study have been shown in the recent paper by Marešovav et al. (2000). Glycoproteins gE, gB, and gH seemed to be produced in similar amounts. The VV-preS2-S-expressing middle envelope protein of Hepatitis B virus was prepared from plasmid pM3 as described previously (Kutinovav et al., 1994). Thymidine-kinase-deficient VV denoted VV TK– is a spontaneous mutant of P13 virus. Human-embryo diploid cells (LEP) and monkey-kidney cells (CV-1) were used for the growth of recombinant viruses. All cells were cultivated in modified medium 199 (EPL) containing bovine serum growth- active proteins but no complete serum (Michl, 1961). The VVs used for immunization experiments were grown in the chorioallantoic membranes of 11-day-old chicken embryos and partially purified by the modified method of Joklik (1962), (Kutinovav et al., 1999). VZV (strain Zuzana) was isolated in our laboratory from skin vesicle of a child with varicella. VZV-Oka was kindly provided by Dr. A. Sauerbrei, Erfurt. Both VZV strains were propagated in LEP cells.
Immunization of mice

Four-week-old outbred female mice, strain CD-1 (ICR)BR (Charles River), were injected intraperitoneally with 0.5 ml PBS containing recombinant VV. Ten mice were immunized in each group. Five weeks later the mice were anesthetized with halothane (Narcolane, Léčiva, Praha) and bled out. Sera used in neutralization test were inactivated at 56°C for 30 min.

Testing of antibodies

ELISA. Levels of VZV- and VV-specific antibodies in individual mouse sera were determined by ELISA as described previously (Ludvíková et al., 1991). For the detection of specific gE and gB antibodies in mice immunized concurrently with these two VZV glycoproteins, pooled sera diluted 1:10 were absorbed prior to testing. The antigens for absorption were prepared from CV-1 cells infected with either VV-gB or VV-gE, and the absorption was performed as described previously (Němečková et al., 1996).

Western blotting. Specific gE and gB antibodies were quantified by Western blotting. In brief, LEP cell cultures infected with VZV were harvested 2 days after infection when the CPE had reached 90%. The cells, washed twice with PBS, were scraped into PBS, centrifuged at 3000 g and lysed in Laemmli buffer with 2-mercaptoethanol. Samples were incubated at 95°C for 5 min and applied on top of a 7.5% PAAG using a wide single-well comb. The proteins separated were transferred to a nitrocellulose membrane using the semidy electrophoretic method. The membrane was stained with Ponceau S and cut into 5-mm strips. The strips, preincubated with 10% non-fat dry milk in PBS for 1 h, were incubated with mixtures of the sera being tested, diluted 1:50, at 4°C overnight. After washing (PBS, 0.2% Tween 20), the strips were incubated with swine anti-IgG labeled with horse-radish peroxidase, washed, and stained with diaminobenzidine. The maxima of the 98-kDa band and the 120-kDa band corresponding to gE and the uncleaved gB molecule, respectively, were scanned and quantified using the Scan PacK 3.0 programme (Biometra).

Radioimmunoprecipitation. Antibodies to gH and gL proteins were determined using immunoprecipitation of [35S]methionine- and cysteine-labeled VZV antigens prepared by the procedure described previously (Němečková et al., 1996). The extracts were prepared from monolayers of CV-1 cells infected with VV-gH/gL or VV-gH recombinant or parental VV. Mixed sera from 10 mice, diluted 1:6, were absorbed with wt VV-infected cells before testing as already described (Němečková et al., 1996). Monoclonal antibody V3 (Sugano et al., 1991), obtained through the courtesy of T. Sugano, Tokyo, was used to detect processed gH in complex with gL and a zoster-patient serum was used to recognize premature gH. Autoradiograms were scanned and density of specific bands (118 and 97 kDa for gH and 19 kDa for gL) was quantified using the Scan PacK 3.0 programme (Biometra).

Neutralization assay

The VZV-neutralizing capacity of sera was examined by a plaque reduction test. The cell-free virus used in the assay was prepared similarly as was described by Ilobi and Martin (1989). In brief, VZV (strain Zuzana)-infected LEP cells exhibiting about 50–70% c.p.e. were scraped into E-MEM supplemented with 5% sucrose, 0.1% sodium glutamate, and 10% thermoinactivated newborn calf serum. The resulting cell suspension was sonicated (Soniprep, MSE) at amplitude 14 μm for 30 s and centrifuged at 950 g for 15 min. The supernatant fluid was used as cell-free virus preparation, and it was stored in aliquots at −65°C. In the neutralization test, the viruses were diluted 1:20–1:30 to achieve 100 ± 50 PFU in control cultures. Several cell-free virus stocks were used in the present study, but the data in each table or figure were always obtained with the same cell-free virus preparation. Equal volumes of virus, serial dilutions of heat-inactivated mouse serum and 1:4 diluted guinea pig serum, as source of complement, were mixed and incubated for 1 h at 34°C. To test complement-independent antibodies, the heat-inactivated (56°C/30 min) guinea pig serum was used in the mixtures. In the control mixtures, the serum tested was replaced by medium. A volume of 0.3 ml of each mixture was inoculated on two 60-mm Petri dishes with LEP cell monolayer at room temperature and 30 min later 5 ml of culture medium (E-MEM, 10% heat-inactivated newborn calf serum) was added. After 10 days of incubation at 37°C, the medium was removed and cells were stained (E-MEM, 1% calf serum, 0.05% neutral red) at room temperature for ≥1 h. Plaques were counted immediately after removal of staining medium. The neutralizing titer was expressed as the reciprocal of the highest serum dilution causing ≥50% plaque number reduction in relation to control.

Skin test in guinea pigs

Groups of three to four outbred female guinea pigs (BFA strain) weighing 225–250 g were injected with two separate doses of 5.106 PFU of recombinant VV in 0.5 ml PBS given simultaneously intraperitoneally and subcutaneously in the back. Control animals received PBS only. One group of animals was injected subcutaneously with 6.106 PFU of extracellular VZV-Oka. Skin tests were performed 18 days after immunization. Skin test antigens were prepared according to the modified method of Kamiya et al. (1977). In brief, the OKA strain of VZV was used for preparation of skin-test antigen. Monolayers of LEP cells with freshly changed medium EPL were inoculated with virus-infected cells at a ratio of one infected cell to four uninfected cells. After incubation at 37°C for
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