Identification of antigenic sites on pseudorabies virus glycoprotein gp50 implicated in virus penetration of the host cell

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Five monoclonal antibodies specific for glycoprotein gp50 of pseudorabies virus were used to make a topographical map of gp50 and to determine the biological function of the different antigenic domains. Three antigenic domains were identified by competition binding assays and additivity assays (IA, IB, II). Domain IA corresponds to a continuous epitope, whereas domains IB and II consist of one or several discontinuous epitopes, identified by their resistance to heat or reducing treatments. Domains IA and IB correspond to sites highly involved in virus neutralization. Neutralization experiments by monoclonal antibodies recognizing domains IA and IB and performed before or after adsorption of virions to cells showed that these domains have a role in penetration of virus into the cell.

Pseudorabies virus (PRV) is responsible for lethal infections in numerous animal species. The viral envelope contains at least seven glycoproteins (Hampl et al., 1984; Rea et al., 1985; Wathen & Wathen, 1984). Among them, glycoprotein gp50 appears for various reasons to be of major importance in antiviral immunity: (i) it is the target of neutralizing monoclonal antibodies (Eloit et al., 1988; Wathen et al., 1985; Wathen & Wathen, 1984); (ii) infection of purified gp50 or of CHO cells synthesizing this glycoprotein were found to protect mice and pigs against a virulent challenge (Ishii et al., 1988; Marchioli et al., 1987); (iii) injection of a recombinant virus vaccine encoding gp50 protects the mouse against a virulent challenge (Marchioli et al., 1987). These data are now well established, but the role of gp50 in the viral cycle has not yet been demonstrated clearly. In order to determine this role, we tried to identify different antigenic domains using a panel of five monoclonal antibodies (Eloit et al., 1988) in competition and additivity assays. The continuous or discontinuous character of these domains was determined by their resistance to the action of different denaturing agents. Moreover, analysis of interference with the virus growth cycle by monoclonal antibodies specific for each domain in some cases enabled their roles to be defined. Using this panel of monoclonal antibodies three antigenic domains were distinguished. Two of them include epitopes which are involved in the penetration of cells by the virus.

Experiments were conducted with anti-gp50 monoclonal antibodies purified from mouse ascitic fluids by gel filtration on Sephacryl S300 (Pharmacia), and concentrated by ultrafiltration (Minicon B125, Amicon). Epitopes of gp50 were mapped initially by competition binding assays with antibodies purified by gel filtration, and sometimes by subsequent column chromatography on DEAE-Tryrsacryl (Laurent et al., 1982). They were labelled with peroxidase using the periodate method (Nakane & Kawoi, 1974) with a peroxidase : IgG molar ratio of 4:1. ELISA microplate wells were coated with a solution of purified viral glycoproteins as previously described (Eloit et al., 1989). The competition experiment involved simultaneous incubation of 100 μl of peroxidase-labelled antibody and two-fold dilutions of each unlabelled monoclonal antibody for 30 min at 37°C. The dilution of the peroxidase-labelled monoclonal antibody was chosen to yield an absorbance reading ranging between 1.5 and 1.9 in the absence of competition. After three washings, the substrate solution (Eloit et al., 1989) was added. The percentage competition was calculated for each concentration of the unlabelled antibody as the ratio of the A recorded for the unlabelled antibody to the A recorded for the same concentration of a control negative antibody. These competition binding assays led to the identification of two groups of antibodies (Table 1). The first group (I) includes the four monoclonal antibodies 22M24, 10X16, 54N13 and 1W10, each of which inhibited the different labelled antibodies at levels equivalent or superior to the homologous competition at similar concentrations. The second group (II) included only antibody 5Q20. The latter did not compete with any labelled antibody except...
1W10 for which a slight inhibition was noticed. On the other hand, no unlabelled antibody inhibited the binding of the labelled antibody 5Q20, except antibody 22M24 which showed slight inhibition at its maximum concentration. Nevertheless, a 32-fold lower concentration of unlabelled antibody 22M24 strongly inhibited binding of the other four labelled antibodies. These results indicate non-specific competition related to steric hindrance in the binding of unlabelled antibodies to the same molecule but at epitopes distinct from that of the labelled antibody. This non-specific competition was not demonstrated with a peroxidase-labelled anti-glII antibody which does not recognize gp50.

These results were completed by mapping epitopes of gp50 by additivity assays, as described by Friguet et al. (1983). Microtitration plates were coated as previously. Unlabelled antibodies were studied after mixing in pairs (100 µl of each antibody). The concentrations of antibodies used were twofold to fourfold higher than those saturating the antigen. The technique is based on the following principle; if a pair of antibodies recognizes distinct epitopes, the absorbance used must be higher (additivity effect) than if they recognize the same epitope (competition effect). For each couple, the additivity was calculated and considered significant if it was higher than 50% (Friguet et al., 1983). Results indicated in Table 1 confirm and complete those of the competition assays. Group 1 can be divided into two subgroups: group IA which contains antibody 10X16 and group IB which contains antibodies 1W10, 22M24 and 54N13. As in the competition assays, this experiment distinguished the antibody 5Q20 of group II. Thus it appeared that the additivity assay was more informative than our competition assays probably because of the steric hindrance produced by the peroxidase conjugation of antibodies in the competition binding assay.

These topographical analyses were completed by the study of the resistance to heat denaturation of epitopes recognized by the different antibodies, either with or without 2-mercaptoethanol (ME) treatment to reduce intramolecular disulphide bridges. Microtitration plates were coated with a solution of purified viral glycoproteins (Eloit et al., 1989). In some experiments the antigen was pre-heated for 3 min at 100 °C with or without 2-5% ME. The wells were then saturated with 3% bovine serum albumin (BSA) for 30 min at 37 °C and washed three times. They were coated with serial dilutions of monoclonal antibodies in phosphate-buffered saline (PBS) pH 7-2, 0-05% Tween 20, 3% BSA and incubated for 30 min at 37 °C. The reaction was developed with rabbit anti-mouse IgG antibodies labelled with peroxidase (Eloit et al., 1989). ELISA titres were expressed as the last dilutions that gave an absorbance greater than or equal to 0-200. Ratios of antibody titres with undenatured antigens to antibody titres with denatured antigen are shown in Table 1. A high ratio corresponds to a high degree of denaturation of the epitope by the corresponding treatment. Three classes of epitopes, which matched the topographical groups, were identified: the first class, corresponding to topographical subgroup IA (antibody

### Table 1. Properties of anti-gp50 antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Competition binding assays*</th>
<th>Additivity assays*</th>
<th>Denaturation†</th>
<th>Neutralization (%)‡§</th>
<th>Neatulation (%)‡§</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X16</td>
<td>IgG1</td>
<td>I</td>
<td>IA</td>
<td>Heat: 2</td>
<td>ELISA: 163840</td>
<td>57</td>
</tr>
<tr>
<td>22M24</td>
<td>IgG2a</td>
<td>1</td>
<td>IB</td>
<td>Heat + ME: &gt; 2048</td>
<td>10240</td>
<td>54</td>
</tr>
<tr>
<td>54N13</td>
<td>IgG1</td>
<td>1</td>
<td>IB</td>
<td>32</td>
<td>1280</td>
<td>54</td>
</tr>
<tr>
<td>1W10</td>
<td>IgG2a</td>
<td>1</td>
<td>IB</td>
<td>64</td>
<td>163840</td>
<td>51</td>
</tr>
<tr>
<td>5Q20</td>
<td>IgG1</td>
<td>II</td>
<td>II</td>
<td>1</td>
<td>5120</td>
<td>ND</td>
</tr>
</tbody>
</table>

* According to competition binding assays of unlabelled and peroxidase-labelled antibodies (Friguet et al., 1983), epitopes were assigned to antigenic domains I (sub-divided into IA and IB) and II.

† A solution of purified viral glycoproteins, non-denatured or denatured by heat treatment at 100 °C for 3 min in the presence or absence of ME was used to coat the microtitration plates. Preparations of purified monoclonal immunoglobulins were then studied in dilution. Binding of antibodies was revealed by a biotin-labelled mouse anti-IgG (H + L) antibody. Each division contains the ratio of ELISA titre of the antibody using undenatured antigen to ELISA titre using denatured antigen.

‡ ELISA titres of the antibodies adjusted to 2 mg/ml. ELISA titres are expressed as the reciprocal of the last dilution that gave an absorbance reading > 0-200.

§ Successive dilutions of purified antibodies adjusted to 2 mg/ml were incubated in the absence of complement with 500 p.f.u. of virus (1 h, 37 °C). The mixture was then plated on a confluent monolayer of Vero cells. After 1 h of contact at 4 °C, then 1 h at 37 °C, the cell layer was washed with PBS and agar medium was added. The titre corresponds to the dilution neutralizing 50% of the input virus.

¶ Five-hundred p.f.u. of virus was treated before or after adsorption to cells with a concentration of antibody able to neutralize about 50% of input virus. Plaques were counted 3 days later and neutralization activities of the antibody were calculated in each case as the percentage of plaques that had been neutralized (0~ corresponds to the number of plaques without antibody treatment). Percentage neutralization after treatment with a non-relevant antibody was less than 5%.

ND, Not done.
10X16) was resistant to heat and ME and probably represents a continuous epitope in the polypeptide chain without dependence on the tertiary structure of the protein. The second class, corresponding to topographical subgroup IB (antibodies 1W10, 22M24 and 54N13), probably includes at least one conformational epitope, destroyed by heat treatment of the protein even in the absence of reducing agents. In group II, antibody SQ20 is specific for another class of epitopes which are heat-resistant but not ME-resistant. These results can be interpreted in two different ways. This epitope may be a continuous epitope, present on the surface of native protein due to structural constraints imposed by disulphide bridges, but not accessible once the protein is reduced. This is unlikely because of the absence of reactivity of the corresponding antibodies after reduction. The other hypothesis suggests a discontinuous epitope, stabilized by at least one disulphide bridge and hence heat-resistant.

Thus glycoprotein gp50 carries continuous and discontinuous epitopes, like glycoprotein gD of herpes simplex virus (HSV) (Eisenberg et al., 1982, 1985; Minson et al., 1986) and like glycoprotein gIV of bovine herpesvirus type 1 (Hughes et al., 1988), a possible homologue of glycoprotein gD. Three continuous epitopes (II, V, VII) were mapped in gD at positions 268 to 287, 340 to 356 and 11 to 19 of the mature protein (Eisenberg et al., 1982, 1985). Comparison with the gp50 sequence published by Petrovskis et al. (1986) shows that these amino acid sequences have no equivalent in gp50. By contrast, comparison of the gp50 and gD sequences allows alignment of the first six cysteines out of seven in both molecules (Petrovskis et al., 1986). Each of these cysteine residues is essential for the stabilization of discontinuous epitopes of gD (Wilcox et al., 1988) and they may have the same role in glycoprotein gp50.

The neutralizing activities of the antibodies were used to look for putative roles of antigenic domains of gp50 in the virus cycle. Each preparation of purified antibody was adjusted to 2 mg/ml and titrated against 500 p.f.u. of virus (Kojnok strain; Table 1). The four antibodies with the most potent neutralizing activity (10X16, 1W10, 54N13 and 22M24) were located in antigenic domain I, whereas antibody SQ20 with very weak neutralizing activity represented antigenic domain II. Since the epitope(s) corresponding to antibodies 22M24, 54N13 and 1W10 show the same kind of resistance to denaturation and belong to the topographical subgroup IB, it was interesting to check whether their different neutralizing activities were due to different avidities for the protein. The same preparation of antibodies, adjusted to 2 mg/ml, was titrated by ELISA (Table 1). Comparison of ELISA and neutralization titres must be done carefully and take into account possible differences in affinity for different immunoglobulin isotypes of the biotinylated mouse anti-IgG (H + L) antibody used for the development of the reaction. This comparison for 22M24 and 1W10, of the isotype IgG2a, showed an approximately 16-fold higher avidity of antibody 1W10, compared to the ratio of neutralizing efficiency between the two antibodies of 128. The discrepancy between ELISA and neutralizing titres does not seem to be large enough to be attributed to the recognition of different epitopes. The same analysis cannot be performed for antibody 54N13 of isotype IgG1, but the comparison with antibody 10X16 of similar isotype shows that it can be classified among antibodies of low avidity, thus explaining its moderate neutralizing titre. It can therefore be assumed that epitopes corresponding to 22M24, 54N13 and 1W10 antibodies should be identical. This assumption is based on the similarities in their properties: recognition of the same antigenic domain (IB), heat sensitivity and comparable neutralizing titres of the corresponding antibodies when related to their respective avidities.

It was shown that gp50 of PRV exhibits sequence homologies with glycoprotein gD of HSV (Petrovskis et al., 1986). Thus, these glycoproteins may have a comparable biological role in the viral cycle. Monoclonal antibodies neutralizing HSV and directed against gD inhibit virus penetration into cells, but do not significantly inhibit virus adsorption to cells (Highlander et al., 1987). The technique described by Highlander et al. (1987) was used to compare the neutralizing capacity of each antibody when added 2 h before or after virus adsorption at 4 °C. If the neutralization mechanism mainly applies to the adsorption stage, virions adsorbed would be resistant to neutralization. On the other hand, if the neutralization occurs at the subsequent penetration stage, some antibodies would neutralize virus already adsorbed to the cells. Table 1 indicates percentages of neutralization of each antibody when added before or after adsorption of the virus to the cells. All the antibodies from groups IA and IB were capable of efficiently neutralizing the virus after adsorption, with an efficiency close to that of neutralization before adsorption. The influence of the antibodies on the penetration rate was also tested by inactivating extracellular virus with an acid buffer at various times during penetration at 37 °C (Highlander et al., 1987). Then 500 p.f.u. of virus was incubated overnight at 4 °C with sufficient antibodies to neutralize 50% of the virus. The mixture was then plated on a monolayer of Vero cells at 37 °C for various times. After incubation, the extracellular virus was inactivated by a citric acid buffer (pH 3) for 1 min and, after washing, cells were overlaid with medium containing agarose. The number of plaques were counted 3 days later. Fig. 1 shows that the
penetration rate was reduced in the presence of each antibody belonging to groups IA (10X16) and IB (22M24, 54N13, 1W10). The effect of 10X16 was more pronounced than that of antibodies from group IB.

Various glycoproteins have been found to be involved in the viral growth cycle. Glycoprotein gIII plays a role in the adsorption of virus to cells (Schreurs et al., 1988). The penetration of gIII deletion mutants into cells seems to be slower than that of wild-type virus (Zuckermann et al., 1989; Mettenleiter, 1989). In contrast, some anti-gIII antibodies do not inhibit penetration of the virus already adsorbed into the cells. Therefore, this characteristic of gIII mutants may be related to an inefficient and slow gIII-independent adsorption (Zuckermann et al., 1989).

The role of the gp50 is currently less well known. It has been reported that an anti-gp50 monoclonal antibody does not inhibit virus adsorption (Schreurs et al., 1988) unless it is used at high concentration (Zuckermann et al., 1989). Moreover, the same monoclonal anti-gp50 antibody inhibits the viral cycle after adsorption to the cells (Zuckermann et al., 1989). The inhibition of HSV adsorption by high concentrations of anti-gD antibodies has also been reported, but at higher concentrations than those neutralizing the virus (Highlander et al., 1987). The present work shows that antibodies from groups IA and IB have a rather similar neutralizing efficiency when exposed to the virus before or after adsorption to the cells, indicating inhibition of the virus growth cycle after adsorption, as already shown for antibodies specific for HSV gD (Highlander et al., 1987) and gIV of bovine herpesvirus 1 (Hughes et al., 1988). Each of these antibodies is capable of reducing the rate of penetration of virus into cells as judged by their resistance to inactivation by an acid buffer. One of them (10X16) includes a continuous epitope the sequence of which is being investigated.

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