Characterization of Complement-Independent Neutralizing Epitopes on Pseudorabies Virus Glycoprotein gp50

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(Received 3 October 1990/Accepted 2 March 1991)

KEY WORDS: glycoprotein, neutralization, pseudorabies.

Aujeszky's disease is an acute disease of swine caused by pseudorabies virus (PrV) belonging to alphaherpesvi-

us of swine. Because of the great economic losses caused by reproductive failure in sow and high mortality in
piglets, control measures of the disease by vaccination have been practiced in many countries. Although several
live and killed virus vaccines have been developed and applied to the disease [5-8], they still carry problems either
in their safety for the other animal species or in protective efficacy. Since a role of vaccine is regarded as
important for the eradication strategies of the disease, an efficient and safe subunit vaccine that can protect pigs
from the disease and have a marker being serologically distinguished from infected animals, is needed. An
approach to the development of subunit vaccine requires the identification of viral antigens responsible for inducing
the protective immune response.

PrV as well as other herpesviruses has several structural glycoproteins that play an important role in the virus
growth and that may be the major antigens eliciting the humoral and cellular immune response in host animals.
Recent studies have revealed that the importance of PrV glycoproteins gIII and gp50 on the cellular and humoral
immune response in pig [13]. PrV glycoprotein gp50 is essential for virus growth, and a major target of neutralizing
antibodies. Furthermore gp50 synthesized in Chinese hamster ovary cells protects pigs from lethal infection with
PrV [7]. However the location of immunogenic epitopes on native gp50 is not well understood.

In this report, we described the production of monoclonal antibodies (MAbs) to PrV and the presence of two
non-overlapping neutralizing epitopes on gp50 of PrV, Yamagata S-81 strain.

The Yamagata S-81 strain of PrV isolated from pig in the first outbreak in Japan [9], was used for this
experiment. The virus was propagated in baby hamster kidney (BHK 21) cells, and assayed in porcine kidney
(PK-15) cells which were grown in Eagle's minimum essential medium (MEM) containing 10% calf serum.
Virus lysate was obtained from purified virus particles. Culture supernatant was concentrated 20-fold by ultrafil-
tration using the hollow fiber system with a mol. wt. cut-off of 100 K (Amicon DC-2, U.S.A.). This prepara-
tion was layered onto 20/50% sucrose discontinuous gradient and centrifuged in an SW28 rotor (Beckman,
U.S.A.) at 25,000 rpm for 1 hr. Viral layer between 20 and 50% gradient was pooled and dialyzed against TN buffer
(20 mM Tris-HCl pH 7.4, 150 mM NaCl). The purified virus was lysed with 1% nonidet P-40 and centrifuged in
an SW60 rotor (Beckman, U.S.A.) at 32,000 rpm for 1 hr. Virus lysate, the supernatant after the centrifugation, was
stored at -80°C until use.

MAbs against PrV were prepared by the method described elsewhere [12]. Briefly, BALB/c mice were
immunized intraperitoneally with the virus lysate mixed with equal part of Freund's complete adjuvant. Further
booster injections were repeated 3 times at every 14 days without adjuvant by the same route. Three days after
the final injection, the splenocytes of mice were fused with P3-X63-Ag8-U1 mouse myeloma cells using 50% of
polyethylene glycol. The hybridomas secreting the anti-PrV antibody were screened by micro-neutralization assay
and indirect immunoperoxidase plaque staining method

11], and cloned by limiting dilution. Ascitic fluids were obtained by intraperitoneal injection of hybridomas into
pristin-primed BALB/c mice. Isotype of MAb was determined by Ouchterlony's method using commercial reagent
(ICN Immunobiological, UK).

The neutralizing activity was measured by the 50% plaque reduction method in both the presence and
absence of guinea-pig complement. Serial two-fold dilu-
tions of ascitic fluid were mixed with half volume of virus and half volume of either guinea-pig complement diluted
to 1:5 or MEM instead of complement. After 2-hr incubation at 37°C, the mixture was infected to PK-15 cell
monolayer in 24-well microplate of 10 mm in a diameter (Falcon, U.S.A.) for 1 hr at 37°C. The cell monolayer was then
incubated for 2 days at 37°C with MEM containing 2% calf serum and 1.5% methy cellulose. The antibody
titer was determined as a reciprocal of the highest dilution at which the number of plaques was reduced by 50%.

Immunoprecipitation was performed as described else-
where [14]. Briefly, PrV-infected PK-15 cells labeled with


35S-methionine were lysed with lysis buffer (0.5% Nonidet
P-40, 0.1 M NaCl and 1 mM p-amidinophenyl methanesul-
fonyl fluoride hydrochloride in 0.1 M Tris-HCl, pH 8.0).
The radio-labeled cell lysate was incubated with MAb for
overnight at 4°C. The viral antigens bound with MAb
were precipitated by addition of Protein A-Sepharose 4B,
and washed with lysis buffer. Final precipitates were
electrophoresed on 10% polyacrylamide gel and exposed to
X-ray film (Kodak X-OMAT, U.S.A.).

Competitive binding assay was performed as follows.
MAbs were purified from culture supernatant of hybrid-
omas. Culture supernatant of hybridomas with serum-free
medium (Kojin Co., Ltd. Japan) was concentrated by

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ultrafiltration membrane of mol. wt. cut-off of 20 K (Advantec, Japan) and purified by ion-exchange chromatography (Mono Q HR 5/5 column, FPLC system, Pharmacia, Sweden). Purified MABs were iodinated with ¹²⁵I by iodogen method. Competitive radioimmunoassay was carried out in 96 well polyvinyl plates at room temperature. Wells were coated with virus lyaste in 0.05 M carbonate buffer (pH 9.6) for overnight at 4°C, and washed with phosphate buffer saline containing 0.05% of Tween 20. Serial three-fold dilutions of MAB started from 100 µg/ml were incubated in the wells for 1 hr. Without washing, appropriate amount (50,000 cpm/25 µl) of ¹²⁵I-labeled MAB was added to the wells. After 1-hr incubation, the wells were washed. The amount of radioactivity bound to individual wells was determined in a gamma counter (Clinigamma, LKB, Sweden).

The list of neutralizing MABs obtained in this experiment was shown in Table 1. These MABs neutralized PrV in various degrees in the presence of complement. Four MABs neutralized PrV equally either in the presence or absence of complement. The others neutralized the virus much more effectively in the presence of complement than in the absence. MABs 1B6 and 4G6 were classified into the IgG1 and neutralized PrV in high titer without complement. This fact suggested that the MABs 1B6 and 4G6 recognized the critical portion on the virion related with the infection of PrV.

For the identification of viral antigens recognized with MABs, immunoprecipitation was performed. MABs 1B6 and 4G6 precipitated the thick band of mol. wt. of 50 K (Fig. 1). MAB 6G11 precipitated the viral protein of mol. wt. of 95 K. The viral antigen precipitated with MABs 1B6 and 4G6 was determined as PrV glycoprotein gp50 by its mol. wt. and fuzzy band. Eliot et al. described that only the PrV glycoprotein gp50 was a target of neutralizing antibodies which did not require complement for virus neutralization [4]. In this study, MABs 1B6 and 4G6 which neutralized PrV without complement were directed to the PrV glycoprotein gp50. In contrast, the gp50 was not detected on the nitrocellulose membrane by immunostaining with these MABs when the viral proteins were denatured with SDS and transferred to the membrane using either western blotting or dot-blotting (data not shown). It was appeared that the MABs 1B6 and 4G6 recognized the conformational epitopes on gp50, since the reactivity of these two MABs with gp50 was lost after denaturing with SDS.

The relationship between the epitopes for MABs 1B6 and 4G6 was determined by competitive binding assay. The result of competitive binding of both MABs was shown in Fig. 2. The binding of ¹²⁵I-labeled MAB 1B6 to

Table 1. Characterization of monoclonal antibodies to pseudorabies virus

<table>
<thead>
<tr>
<th>MAB</th>
<th>Isotype</th>
<th>Neutralizing titer + Complement</th>
<th>Neutralizing titer - Complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B6</td>
<td>IgG1</td>
<td>2,560</td>
<td>1,280</td>
</tr>
<tr>
<td>4G6</td>
<td>IgG1</td>
<td>10,240</td>
<td>10,240</td>
</tr>
<tr>
<td>14D</td>
<td>IgG1</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>8C4</td>
<td>IgM</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>9A</td>
<td>IgG2b</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>6G1</td>
<td>IgG2b</td>
<td>5,120</td>
<td>80</td>
</tr>
<tr>
<td>19B</td>
<td>IgG1</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>2C4</td>
<td>IgG1</td>
<td>80</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Fig. 1. Autoradiogram of PrV proteins immunoprecipitated with MAB 1B6 (●) and MAB 4G6 (▲). Whole viral proteins (V).

Fig. 2. Competitive inhibition of binding of ¹²⁵I-labeled MAB 1B6 (● and ○) and MAB 4G6 (▲ and △) to PrV glycoprotein by unlabeled homologous (black) and heterologous (white) MAB.

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virus lysate was markedly inhibited by pre-incubation with homologous MAb, however it was not affected by pre-incubation with MAb 4G6. In contrast, the binding of labeled MAb 4G6 to virus lysate was competed only with unlabeled MAb 4G6. The results demonstrated the presence of two non-overlapping epitopes on PrV glycoprotein gp50. The presence of at least three overlapping epitopes on gp50 of the Indiana-Funkhauser strain of PrV has been described by Cee et al. [1] and Wathen et al. [14]. These epitopes were identified by using neutralizing MAbs which required complement for neutralization. In the present study, MAbs 1B6 and 4G6 independently reacted with different epitopes on gp50 and furthermore did not require complement for the neutralization of PrV. The difference of the epitopes recognized with the MAbs demonstrates the presence of at least two distinct sites which contribute to the role of gp50 on the infection, though it is unclear whether two complement-independent neutralizing epitopes are common among PrV strains including the Indiana-Funkhauser strain. The PrV glycoproteins have been found to have partial amino acid sequence homology with herpes simplex virus (HSV) glycoproteins. The glycoprotein gp50 of PrV is homologous to the glycoprotein gD of HSV [10]. The location of neutralizing epitopes on gD of HSV has been previously described [2, 3]. Among eight neutralizing epitopes on gD, four were discontinuous epitopes which lost its reactivity to neutralizing MAbs under the denaturalized condition. The MAbs reacted with such discontinuous epitopes on gD had several important biological properties, including virus neutralization and inhibition of cell-to-cell fusion. In view of homology of PrV glycoproteins to those of HSV, gp50 of PrV also seems to possess the discontinuous epitopes which have the same function as gD of HSV. Because of the failure of reaction of MAbs 1B6 and 4G6 to gp50 in immunostaining after the western blotting, the epitopes recognized with both MAbs might be discontinuous epitopes. Further characterization of these epitopes by using the resistant mutants seems to be needed. These MAbs are regarded as useful for the study of biological and immunological functions of gp50.

ACKNOWLEDGEMENTS. We are grateful to Dr. T. Onodera and Dr. K. Sekikawa, National Institute of Animal Health, for their suggestions and encouragements in this study.

REFERENCES