Protection from Pseudorabies Virus Challenge in Mice by a Combination of Purified gII, gIII and gVI Antigens

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ABSTRACT. The antigens gII, gIII and gVI were purified from the lysates of the pseudorabies virus (PRV) -infected HmLu-1 cells using Sepharose 4 B coupled with MABs against these antigens. Mice immunized with either gII, gIII, gVI antigen or a mixture of them were challenged intraperitoneally with 8.5×10^3 plaque forming units of PRV. All the mice immunized with 1.5 and 4.5 μ g of the mixture and 4.5 μ g of the gIII antigen survived. The sera of mice immunized with the mixture had virus neutralizing activity which was independent of complement, hemagglutination inhibition activity, and recognized major (93 kilodaltons) and minor (129, 74, 68 and 50 kilodaltons) PRV proteins under reducing conditions in western blotting. The serological activity levels in the lower survival group were not different from those in the complete survival. These results indicate that the mixture of each glycoprotein is more effective for eliciting of protective immunities in mice and that serological activity do not always correlate with protection.—**KEY WORDS:** gII, gIII, gVI, PRV.

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Pseudorabies virus (PRV) causes severe acute infection in piglets. Some PRV glycoproteins induce an immune response in the host animal which protects them against PRV infection [4, 14, 21]. When mice and pigs immunized with these glycoproteins are challenged with a lethal dose of PRV, they show high survival rates [8, 12]. Glycoprotein gIII is reported to be an effective vaccine component because it induces virus-specific cytotoxic T lymphocytes (CTL) [20]. Mice immunized passively with monoclonal antibodies (MABs) against gIII are protected against PRV challenge [11, 15]. Glycoprotein gVI (gp50) is effective against PRV infection, as demonstrated by passive and active immunization experiments [8, 11, 15] and by studies with recombinant gVI [6, 10]. Recently, gI, which is concerned with the expression of virulence with gV (gp63) and gIII, has been reported to produce protective antibodies [7]. Although an effective and protective virus component has been defined, little is understood about what is actually required to induce the appropriate immune response against PRV infection. There have been very few experiments on the protective effects of PRV glycoproteins and a mixture of them.

We reported previously the protective effects of gII and gIII which were purified by affinity chromatography with MABs against these antigens [12]. However, the gVI antigen was not tested in previous examination. In an effort to define which of gII, gIII or gVI is more effective against PRV challenge,

immunization experiments with a mixture of purified gII, gIII and gVI antigens were performed. This report will provide some informations concerning the protective effects of these glycoproteins.

MATERIALS AND METHODS

With the following exceptions, the materials and methods, production and cloning of MAB-secreting hybridoma cells, virus neutralizing (VN) test, hemagglutination (HA) -inhibition (HI) test, indirect immunofluorescent assay (IFA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and immunoprecipitation, were performed as previously reported [11].

Monoclonal antibodies against gVI: The selection of hybridoma cells secreting MABs against gVI of PRV IWATE strain was performed by western blotting under nonreducing conditions. MABs reacting with the 50 kilodalton (Kd) protein of the infected-cell lysates were selected. They were identified by immunoprecipitation whether they recognized gVI glycoprotein. MABs were purified from the supernatants of the hybridoma cells by affinity chromatography with Protein-A affi gel (Bio Rad Ltd) and stored at -80°C before use.

Purification of PRV glycoproteins: Specific antigens were purified by affinity chromatography on Sepharose 4 B (Pharmacia) coupled with MABs which recognized gII, gIII or gVI. HmLu-1 cells infected with PRV were dialysed with 0.025 M Tris

Tricin (TT buffer, pH 8.2) containing 0.2% Nonidet-P 40 (NP-40) and were applied to the sepharose coupled with MAb. Then the sepharose was treated with 4 M guanidine hydrochloride (pH 3.5) to elute the specific antigen. The purified antigens were analyzed by SDS-PAGE.

Protection in mice immunized with the purified antigens: Four-week-old ddY mice were immunized and challenged. The mice were divided into five groups; groups 1–3 were immunized with gII, gIII or gVI, group 4 with a mixture of these purified antigens in the same protein concentrations and control group with PBS supplemented 0.1% NP-40. Three volumes of these immunoagents were mixed with seven volumes of oil adjuvant (OAd). A 1.5 μ g and 4.5 μ g of the immunoagents were administered by subcutaneous injection twice at two weeks interval. The mice were then challenged by an intraperitoneal injection with 8.5×10^3 plaque forming units of PRV three weeks after the second immunization. The number of surviving mice was recorded daily for two weeks after the challenge. The immunization and challenge experiments were evaluated by the survival rates of the mice and the serological levels of the VN and HI activities. In addition, sera obtained from three mice selected at random from each group were tested for reactivity the PRV protein by western blotting.

Statistical methods: Differences in survival of different groups in this immunization experiment were analyzed for statistical significance by the chisquare test.

RESULTS

Characterization of MAbs against PRV glycoproteins: Table 1 summarizes the features of MAbs against PRV up to this point. Type A MAbs which precipitated gII glycoprotein had complement-dependent or independent VN activity, and type B MAbs which precipitated gIII glycoprotein had complement-independent VN activity and HI activity [12]. The proteins precipitated by type C MAbs were not identified (data not shown). The type D MAbs established in this experiment precipitated a 50 Kd glycoprotein from the infected-cell lysates (Fig. 1), showing VN activity. They recognized surface antigens of infected-cells in IFA. Therefore type D MAbs were considered to recognize glycoprotein gVI of PRV as described by Eloit *et al.* [5] and Wathen and Wathen [19].

Immunoagents: Purified antigens were prepared from lysates of the PRV-infected HmLu-1 cells using the sepharose coupled with PR-M2 MAb, PR-M41 MAb or PR-M71 MAb. The hybridoma cells which secrete these MAbs had more proliferative capability than others. The proteins contained in the purified antigens were electrophoretically separated on 10% sodium dodecyl sulfate polyacrylamide gels under reducing conditions with 2-mercaptoethanol (Fig. 2). Six μ g of these antigens was applied to the gels. The gII antigen purified by the PR-M2 MAb-coupled sepharose showed three bands at 129, 68 and 60 Kd. The gIII antigen purified by the PR-M41 MAb-coupled sepharose

Table 1. Characterization of MAbs against PRV-glycoproteins

Type	Immunoprecipitation (Kd)	VN activity ^{a)}	Surface antigen ^{b)}	PRV-MAb (PR-M) No.	Identification
A	129/68/60 (140) ^{c)}	— +(C) +	— + +	2, 7 31, 33, 36, 48~50 5, 51	gII
B	93/74	+ +	+ +	17, 19, 39 (HI —) ^{d)} 40, 41, 42 (HI +)	gIII
C	115/100/93	+(C) —	+ +	23 16, 21, 27, 28, 43~45	— ^{e)}
D	50	+	+	67~70, 71, 72~74	gVI

a) The VN activity was evaluated by 50% plaque reducing assay in the presence (indicated as C) or absence of sera of guinea pigs as a source of complement.

b) Obtained by IFA of the unfixed antigens.

c) Obtained under unreducing condition.

d) The HI activity is from our previous report.

e) Unknown.

Fig. 1. Immunoprecipitation of MAbs against gVI of PRV. Immunoprecipitation was performed as described previously [12]. Briefly, ^{14}C -glucosamine-labeled HmLu-1 cells were collected 48 hr after PRV inoculation. After incubating in PBS containing 2% NP-40 at 4°C for 30 min, the supernatants were immunoprecipitated with PR-M71 MAb. Precipitates treated with (+) or without (-) 2-mercaptoethanol were analyzed by SDS-PAGE.

showed two bands at 93 and 74 Kd. The gVI antigen purified by affinity chromatography with the PR-M71 MAb-coupled sepharose showed one band at 50 Kd. In western blotting for identification of the gVI antigen, PR-M71 MAb bound the 50 Kd protein under nonreducing conditions but not under reducing conditions (Fig. 3).

Serological activities and specificities of sera from immunized mice: Before the challenge, serological activities of the immunized mice were tested (Table 2). Sera from group 1 mice immunized with the gII antigen had complement-dependent VN activity and no HI activity. Sera from group 2 mice immunized with the gIII antigen showed complement-independent VN activity and HI activity, except for that one serum showing complement-dependent VN activity. Sera from group 3 mice immunized with the gVI antigen had complement-independent VN activity and no HI activity. Sera from group 4 mice which were immunized with the mixture of antigens had the same serological activities as those of group

Fig. 2. SDS-PAGE analysis of the purified antigens. Purified antigens were obtained from lysates of PRV-infected HmLu-1 cells and were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol. The gII (lane 1), gIII (lane 2) and gVI (lane 3) antigens were purified by affinity chromatography on Sepharose 4 B coupled with PR-M2, PR-M41 and PR-M71 MAbs, respectively.

Fig. 3. Western blotting of gVI antigen. The gVI antigen purified from cell-lysates was analyzed by western blotting using PR-M71 MAb with (+) or without (-) 2-mercaptoethanol.

Table 2. Immunization with purified antigens in mice

Group	Immuno-agent	Dose (μ g)	Before challenge ^{a)}			Protected/ tested (%)	p ^{b)}
			VNc-	VNc+	HI		
1	The gII antigen	1.5	<0.35	2.34~3.19	<8	9/11 (82)	<0.01
		4.5	<0.35	2.07~3.56	<8	9/11 (82)	<0.01
2	The gIII antigen	1.5	<0.35~2.45	1.22~1.56	<8	8/11 (73)	<0.5
		4.5	1.68~1.80	2.32~3.65	≥ 64	11/11(100)	<0.01
3	The gVI antigen	1.5	2.77~3.41	2.72~3.29	<8	6/11 (55)	>0.5
		4.5	2.51~3.18	2.49~3.33	<8	8/11 (73)	<0.5
4	The mix antigen	1.5	1.72~2.85	2.05 3.02	<8~32	11/11(100)	<0.01
		4.5	2.22~2.95	2.51~3.40	8~32	11/11(100)	<0.01
Control	NP-40 PBS		<0.35	<0.35	<8	4/16 (25)	

a) VN titer is expressed as index (log10) and HI titer is expressed as the reciprocal of the highest serum dilutions that showed complete HI. VN test was performed in the presence (VNc+) or absence (VNc-) of fresh guinea pig serum as a source of complement.

b) P value for the difference in survival between the immune group and the control group.

Fig. 4. Proteins recognized by the sera of mice immunized with the purified antigens in western blotting. PRV proteins recognized by the sera of mice immunized with the purified antigens were analyzed by western blotting: lane 1, the sera of group A (immunized with the gII antigen); lane 2, group B (immunized with the gIII antigen); lane 3, group C (immunized with the gVI antigen); lane 4, group D (immunized with the mixture); lane 5, group E (controls).

2 mice. The VN titer of groups 1, 3 and 4 had no correlation with dose, whereas, HI titer of sera from group 2 correlated to dose and survival rate. Sera from control group had no serological activities.

Subsequently, the sera before the challenge were analyzed by western blotting to determine which proteins were recognized. The purified PRV from

the infected-HmLu-1 cells was used as an antigen under reducing conditions. Sera of group 1 mice recognized major 129, 68 and minor 60 Kd proteins. Group 2 mice recognized major 93 and minor 74 Kd proteins. Group 3 mice recognized the major 50 Kd protein and group 4 mice, major 93 and minor 129, 74, 68 and 50 Kd proteins of PRV (Fig. 4), as shown in Fig. 2. Control sera showed no reactions.

Survival of immunized mice against PRV challenge: Mice immunized with these antigens were protected against PRV challenge. From the survival rate, most protective effects were found in group 4, at 1.5 and 4.5 μ g doses, and in group 2, at a 4.5 μ g (Table 2). Their survival rates were 100%. The survival rate of group 1 mice was 82% and that of group 3 mice was about 55 or 73%. The differences in the survival rate between immunized groups except for group 3 at 1.5 μ g dose and control group were statically significant. The challenge dose was sufficient to kill 25% of control mice.

DISCUSSION

We established MAbs against the PRV glycoproteins, gII, gIII, gVI and an unknown antigen recognized by type C MAbs. The analysis of the characterization of MAbs suggests that gII has variety of antigenic sites, like gB of herpes simplex virus (HSV) described by Mester *et al.* [13], which was reported to have homology with gII of PRV at the DNA and amino acid levels [16]. Glycoprotein gIII is involved in VN site which has HA activity and

gVI induces the antibodies which have complement-independent VN activity.

Although PR-M71 MAb did not bind gVI antigen under reducing conditions, sera from group 3 mice immunized with the gVI antigen bound the 50 Kd protein under reducing conditions in western blotting (Fig. 2) and had more intense VN activity than those from other groups (Table 2). These results show that type D MAbs recognize specifically gVI and suggest that the sites recognized by type D MAbs should be concerned with the disulfide bonds. Petrovskis *et al.* [15] reported that gVI has conserved cystein residues in the amino acid sequence and suggested that this conservation indicated the probability of the disulfide linkages in gVI.

We found that gII and gIII antigens induced a protective immunity in mice which did not correlate to the titers of the VN, HI and ELISA activity [12]. However the protective effect was not observed in the antigen recognized by type C MAbs (data not shown). Those examinations did not compare the protective effects of gII and gIII glycoproteins.

In HSV studies of immunization experiments with recombinant gB and gD or a mixture of HSV glycoproteins, definite difference in protection against HSV infection was not suggested, although the advantages of immunization with gD or the mixture were shown [2, 17]. In the passive immunization experiment with MAbs against HSV glycoproteins, the mixture of five MAbs showed more protective effect than one MAb [1].

Comparing the protective effects of the gII, gIII, gVI antigens and the mixture, the mixture was found to be the best protective immunoagent since no mice immunized with the mixture died. Mice immunized with 4.5 μ g of the gIII antigen survived completely. Moreover, the sera of group 4 recognized a major 93 Kd protein, which was identified as the gIII component. Therefore, these results indicate that the gIII antigen confers upon mice more advantageous protection against PRV infection than the gII and gVI antigens. On the other hand, the survival rate in group 3 immunized with the gVI antigen was unexpectedly low. The mice immunized with 1.5 μ g of the gVI antigen did not survive significantly compared with control mice. Protection by gVI against PRV has been reported in mice and pigs [8, 18]; gVI synthesized in cells induced a protection against lethal PRV infection in mice and pigs [10]. Moreover, gVI expressed on a defective adenovirus vector was reported to partly protect

mice and rabbits from PRV challenge [6]. Since dose response was found in group 3 in this examination, more gVI antigen may increase the survival rate of the immunized mice. Our results seem to be attributable to the incomplete purification of the gVI antigen in contrast with the others.

The gII antigen induced mice to produce VN antibodies requiring complement, the gIII antigen produced VN and HI antibodies and the gVI antigen produced a high titer of VN antibodies which were complement-independent. No correlation between the serum antibody titer and the survival rate was found. Group 2 showed correlation between the HI titer and the survival rate, but HI titer of them was higher than that of group 4 which showed 100% survival. Therefore, ultimately the protective effect observed in mice immunized with the antigen mixture is due to the interaction of these antigens, each of which played a different role *in vivo* inducing the immune response, probably in a cell-mediated manner rather than humoral immunity. This suggests that the serum antibodies measured in this study do not directly indicate protective ability.

When we examine purified antigens for immunogenicity, the purification level has to be defined. In this study, we analyzed the purified antigens using SDS-PAGE and adjusted their protein concentrations by the method of Lowry [9]. However, the purification level of these antigens was not same. Exact quantification of the immunoagents is complicated. We suggest that the quantification of immunoagents should be determined by serological activities.

We focused upon the survival rates of the immunized mice in this study. It remains to be resolved whether this combination of antigens is available for pigs as well as in mice and whether these antigens can inhibit excretion and latency of PRV in host animals. HSV type 1 gD was effective for preventing latency, but not completely [3]. PRV glycoproteins in pigs remain to be evaluated.

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