Characterization of Protective Viral Glycoproteins for Pseudorabies Virus Infection

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Pseudorabies virus (PRV) glycoproteins have revealed to have various characteristics. Viral glycoproteins gII, gIII and gp50 possess epitopes which induce antibodies with virus neutralizing (VN) activity [3, 4, 14]. Glycoproteins, gII and gp50 are essential for PRV replication, but gIII is not directly involved [15]. Glycoprotein gIII plays a major role in the adsorption of the virus to host cell receptors [11, 12]. The nonessential glycoproteins gI, gp63 and gIII have been shown to affect virus release in conjunction with one another [17]. Glycoproteins, gIII and gp50 induce high protection in mice and pigs against PRV infection by passive immunization with the monoclonal antibody (MAb) or by active immunization with the purified antigen [3, 5, 10, 14]. Moreover, gp50 was evaluated as a subunit PRV vaccine [9, 16]. On the other hand, MAbs against gII were partially effective against PRV in pigs immunized passively but not in mice [10]. Ben-Porat *et al.* [2] reported that gIII showed a high level of antigenic drift, although no drift was observed for gII. Which glycoproteins, gII, gIII or gp50 is essential for the PRV vaccine is unclarified.

Recently, Tetsu *et al.* [13] reported that PRV possessed a hemagglutination (HA) antigen for mouse erythrocytes and that pigs infected with PRV produced HA-inhibition (HI) antibodies. However, the HA antigen has not yet been identified and the relationship between HI antibodies and protection against PRV infection is not known.

We have been studying PRV proteins in an effort to elucidate their protective effect against PRV infection.

We studied the protective properties of the PRV glycoproteins, gII and gIII, in PRV infections and their characteristics with HA activity towards mouse erythrocytes.

The IWATE strain of PRV was used. The seed virus was propagated in chicken embryo fibroblasts and in HmLu-1 cells from hamster lung. The virus was routinely grown in HmLu-1 cells or in RK-13 cells from rabbit kidney. These cells were grown in Eagle's MEM containing 5% fetal calf serum (FCS) and 10% triptose phosphate broth.

MAbs secreting specific antibodies against PRV proteins were prepared according to the method of Ando [1]. The spleen cells of BALB/c mice which were immunized with purified PRV grown in RK-13 cells or infected-cell lysates with oil adjuvant (OAd) by subcutaneous (s.c.) injection were fused with X63-Ag8-653 mouse myeloma cells. Positive hybridoma cells were selected by screening them with an enzyme-linked immunosorbent assay using cell lysates infected and mock-infected with PRV as the antigens. They were cloned 4 times by the limiting dilution method. MAbs were purified by affinity chromatography with Protein-A affi gel (Bio Rad) from ascites fluid which was produced by mice injected intraperitoneally (i.p.) with hybridoma cells or culture medium before use. The MAbs, PR-M2 and PR-M41, were characterized by VN test and indirect immunofluorescent assay (IFA) (Table 1). The VN activity was assayed by plaque-reducing activity using RK-13 cells in the presence or absence of fresh guinea pig serum as a source of complement. IFA was performed by two techniques: HmLu-1 cells infected or mock-infected with PRV which were fixed with cold acetone were used for cytoplasmic IFA and HmLu-1 cells not fixed were used for surface IFA. PR-M2 MAb did not show the VN activity and did not detect antigens by surface IFA, but PR-M41 MAb showed the VN activity and detected antigens by both IFA techniques. By means of immunoprecipitation using virus-infected HmLu-1 cells labelled with ¹⁴C-glucosamine, PR-M2 MAb precipitated

Table 1. Characterization of PR-M2 and PR-M41 MAbs

MAb	Specificity ^{a)} (kilodalton)	Isotype	VN ^{b)}		IFA ^{c)}		HI activity ^{d)}
			+c	-c	cytoplasmic	surface	
PR-M 2	129/68/60(140) ^{e)}	G _{2a}	-	-	+	-	not tested
PR-M41	93/74	G ₁	+	+	+	+	+

a) Classification according to reaction type in immunoprecipitation.

b) Virus neutralization tests were performed by plaque-reducing assay. +c: with complement, -c: without complement.

c) The fixed antigens were used in cytoplasmic IFA and the unfixed antigens were used in surface IFA.

d) The materials purified with PR-M41 MAb and the culture supernatants of cells infected with PRV were used as HA antigens.

e) Molecular weight of the immunoprecipitated protein under reducing conditions (under nonreducing conditions).

Fig. 1. Immunoprecipitation of ^{14}C -glucosamine-labeled PRV glycoproteins with MAb. Radiolabeled cells were prepared by infecting HmLu-1 cells with PRV labeled with 111 KBq/ml of ^{14}C -glucosamine at 60 min post-inoculation (PI). After being washed with PBS at 48 hr PI, labeled cells were treated with PBS containing 2% Nonidet P (NP)-40 at 4°C for 30 min. After centrifugation, the supernatant was immunoprecipitated with PR-M2 MAb (I) and PR-M41 MAb (II) and the pellets treated with 2-mercaptoethanol (+) or untreated (-) were analyzed by SDS-PAGE.

140 kilodalton (Kd) protein under nonreducing conditions and 60, 68, 129 Kd under reducing conditions and PR-M41 MAb precipitated 74 and 93 Kd proteins (Fig. 1). From the results of these assays, the disulfide-linked PRV glycoproteins recognized by PR-M2 MAb were defined as gII [3, 4, 8]. The non-disulfide-linked PRV glycoproteins recognized by PR-M41 MAb were defined as gIII [3, 4, 12].

For the identification of protective viral proteins, the gII and gIII antigens were purified from the cell lysates by affinity chromatography with PR-M2 and PR-M41 MAb. RK-13 cells infected with PRV at m.o.i. of 1 to 5 ($\text{TCID}_{50}/\text{cell}$) were harvested at 48 hr post-infection. The cell pellets were washed with 0.025 M Tris Tricin (TT buffer, pH 8.2) and stirred with TT buffer containing 0.2% NP-40 at 4°C overnight. After centrifugation, the supernatant was applied to a Sepharose 4B (Pharmacia) column coupled with MAb. The impurities were removed by washing them with 10 bed volumes of PBS. Elution was performed with 3 M potassium thiocyanate (pH 7.2) or 4 M guanidine hydrochloride (pH 3.5). The eluted materials were concentrated and dialyzed in PBS and assessed by the method of Lowry [7]. The purified proteins were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [6] in separating gels containing 10% acrylamide monomer cross-linked with bisacrylamide and the west-

ern blot technique. Figure 2 shows the results for the materials purified with PR-M2 and PR-M41 MAb. The PR-M2 materials showed mainly one band (140 Kd under nonreducing and 68 Kd under reducing conditions) (Fig. 2, I-A), while the PR-M41 materials showed mainly two bands of 93 and 74 Kd (Fig. 2, II-A) with Coomassie Brilliant Blue (CBB) staining. These bands reacted with concanavalin A (Con A) by using the avidin-biotin peroxidase complex (Vecter Ltd.) in western blots (Fig. 2, B-a). A 68 kilodalton protein purified with PR-M2 MAb reacted with the homologous antibody and with serum from a pig immunized with PRV infected-cell lysates (Fig. 2, I-B-b, c). Seventy-four and 93 kilodalton proteins purified with PR-M41 MAb also reacted with the homologous antibody but only slightly with pig serum (Fig. 2, II-B-b, c). Therefore, the glycoproteins in the PR-M2 materials were defined as gII and the PR-M41 materials as gIII. Moreover, the HA activities of these purified antigens and the HI activity of MAb were tested. HA and HI tests were carried out by the microtiter method [13] with slight modifications. The diluent used was PBS containing 0.1% BSA and 0.01% gelatin. Erythrocytes obtained from BALB/c mice were fixed with PBS containing 1% glutaraldehyde and were used in a 0.5% suspension. Although the gIII antigen purified with PR-M41 MAb showed HA activity, the gII antigen purified with PR-M2 did not. In the HI test, PR-M41 showed HI

Fig. 2. SDS-PAGE with CBB staining (A) and western blots (B) of the materials purified with PR-M2 MAb and PR-M41 MAb from PRV-infected RK-13 cell lysates. The PR-M2 materials (I) were eluted with 3 M potassium thiocyanate and the PR-M41 materials (II) were eluted with 4 M guanidine hydrochloride. These materials were analyzed by SDS-PAGE with (+) or without (-) 5% 2-mercaptoethanol. The nitrocellulose membranes onto which they were transferred by western blots were incubated with Con A (a), homologous MAb (b), or pig immune serum (c). After being washed PBS contained 0.05% Tween-20, the membranes were incubated with the avidin-biotin peroxidase complex or peroxidase-conjugated antiserum (Cappel Ltd.). The bound enzyme was located with 3,3'-diaminobenzidine and H_2O_2 .

Table 2. Protection against PRV infection in mice immunized with purified protein from PRV infected cell lysates

Immunizing agent ^{b)}	Antibody titer before the challenge ^{a)}			The challenge with PRV	
	VN ⁺ ^{c)}	VN ⁻ ^{c)}	HI	Survivors/total	%
gII antigen	2.88	0.95	<16	9/10	90
gIII antigen	2.76	1.96	256	10/12	83
NP40-PBS	<0.35	<0.35	not tested	0/10	0

a) Antibody titer 3 weeks after the second immunization.

b) Immunizing agent were injected with oil adjuvant.

c) VN: virus neutralization test ($\log_{10}$), +c: with complement, -c: without complement.

activity with the gIII antigen and PRV (Table 1).

These purified antigens were used for an active immunization of the mice. Four-week-old ddY mice were immunized with 10 μ g of the purified antigens with OAd

by s.c. injection, and then boosted 2 weeks later. Three weeks after the second immunization, the animals were challenged with 50 LD₅₀ of PRV by i.p. injection. Control mice for challenge experiments were injected s.c. with

Fig. 3. The PRV proteins recognized with the mouse immune sera by western blots. The nitrocellulose membranes onto which they were transferred electrophoretically the homologous immunogen (a) and infected-cell lysates (b) treated with 2-mercaptoethanol (+) or untreated (-) were incubated with the sera from mice which were immunized with the gII antigen (I) and the gIII antigen (II) and which were obtained before the challenge experiment. The proteins reacted with these sera were detected as described in a footnote of Fig. 2.

PBS containing 0.1% NP-40 with or without OAd. The number of dead mice was recorded daily for 2 weeks after the challenge. The survival rate of mice immunized with 10 μ g of the gII and the gIII antigens was 83–90% (Table 2). No control mice survived when challenged with PRV. Antibodies against PRV in sera of these mice before the challenge were detected by the VN test and HI test. The VN activity of sera from mice immunized with the gII antigen was dependent on complement and it had no HI activity. On the other hand, that with the gIII antigen was found to be independent of complement and it had HI activity. These immune sera were also analyzed by the western blot (Fig. 3). The sera from mice immunized with the gII antigen recognized a 140 Kd protein of infected-cell lysates under nonreducing conditions and 68 and 129 Kd proteins under reducing conditions, but they recognized one band of the gII antigen, as we obtained in the analysis of the gII antigen using PR-M2 MAb by western blots (Fig. 2). The sera of mice immunized with the gIII antigen recognized 74 and 93 Kd proteins by the homologous immunogen and the infected-cell lysates.

In passive immunization experiments conducted by other workers, the glycoproteins gIII and gp50 are effective for PRV immunization [5, 10, 14], and gII had a partial protective effect [10]. By contrast, we have observed that gII and gIII provide a protective effect against PRV challenge in mice.

However, the present findings suggest that the protection against PRV infection is not sufficient for mice immunized with either gII or gIII.

This study showed that gII induced the intensive production of complement requiring VN antibodies and

that gIII induced the production of complement nonrequiring VN antibodies which showed HI activity. Zuckerman *et al.* [18] identified gIII as one of the major proteins eliciting a cytotoxic T lymphocyte response to PRV in mice and pigs. They further recommended that gIII should be included as a component of the PRV vaccine.

Schreurs *et al.* [12] reported that gIII was concerned with the adsorption of PRV to the host cells. Our findings, therefore, imply the possibility that the VN activity of the anti gIII antibody may inhibit the adsorption of PRV and that the HA site may be concerned with this adsorption. It remains to be elucidated whether the protection of PRV is related to HI antibodies.

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