Induction of Neutralizing Antibodies by Structural Proteins VP1 and VP2 of Swine Vesicular Disease Virus

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(Received 7 July 1986/Accepted 30 October 1986)

Jpn. J. Vet. Sci. 49(1): 129-132, 1987

KEY WORDS: ELISA, swine vesicular disease virus, VP2.

Picornaviruses contain a single molecule of single-stranded RNA, which acts as a messenger RNA, specifying information of the synthesis of nonstructural proteins and structural proteins. The same RNA molecule also initiate replication *in vivo*. When, as with picornaviruses, the genome serves as a single messenger RNA, a giant polyprotein is made and then this protein is cleaved to yield several viral proteins. Cleavage is carried out by specific virus coded enzymes which recognize the secondary and tertiary structures of polyprotein and other precursor proteins. A second cleavage produces three structural proteins (VP0, VP3, VP1). A third cleavage of precursor capsid polypeptides (VP0) occurs during the virion maturation which produces two structural protein (VP2, VP4) [6].

VP1 protein is responsible for the induction of neutralizing antibodies in foot-and-mouth disease [1], while VP2 is responsible in coxsackievirus B3 [2]. However there is not enough information in the responsible structural proteins for the swine vesicular disease virus (SVDV) neutralizing antibody induction.

Since SVDV is neutralized by the antibody to coxsackievirus B5, and SVDV can also infect man, there was speculation about the relationship between SVDV and coxsackievirus B5 [3, 7]. Although two viruses were differentiated by neutralization test, there have been considerable diversity in the antigenicity in SVDV and coxsackievirus B5 [5, 7]. These diversity prompted a investigation of biochemical analysis of structural protein, and role of structural protein for the induction of neutralizing antibodies [7]. In this report experiments were carried out, as a part of project to develop the synthetic vaccine, to identify the viral protein or polypeptides which were responsible to induce neutralizing antibody in picornaviruses.

Japan 1/73 [8] strain of SVDV grown in IB-RS-2 cell monolayers was concentrated by

ultrafiltration using Amicon filter system (DC-2, 100,000 M.W. cut off), and was purified with sucrose gradient (15-45% (w/v) sucrose in 0.15 M NaCl, phosphate buffered saline (PBS), pH 7.6 at 25°C) in 80,000 G for 2.5 hr at 10°C. The peaks of virus or empty particles were pooled, diluted with 0.15 M NaCl, or PBS, precipitated with 2 volumes of acetone at -20°C overnight. The precipitate was deposited by centrifugation, dried, resuspended in disruption buffer (0.0625 M Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 2% beta-mercaptoethanol and 10% (w/v) glycerol) and analyzed on high resolution polyacrylamide gel (PAGE) slabs using Tris-glycine buffer. The main gel (12.5%) and the stacking gel (5%), modified to contain 0.5 M urea, were subjected to electrophoresis for 4-6 hr at 100 V. The gel was stained with 0.25% (w/v) Coomassie blue in methanol-acetic acid (2:1), destained in methanol-acetic acid, dried under vacuum on to wettable cellophane.

As shown in Fig. 1, well defined viral proteins were obtained using SDS-PAGE with Tris-glycine buffer system [7]. Protein VP0, the precursor of VP2 and VP4, was found in empty particles and also was found in small amounts in full particles of SVDV. The molecular weights of VP0, VP1, VP2 and VP3 were 37K, 33K, 29K and 32K, respectively. Each protein band was dissected from the gel, mixed with Freund's complete adjuvant, and injected subcutaneously and intramuscularly into 3 months old guinea pigs. Mobility of VP2 was clearly different from that of VP3, although these proteins were reported to be not well separated in Hong Kong 36/71 and France 1/73 strains of SVDV [7].

In another procedure, the peaks of virus particle or empty particles were dissociated by 3 M or 8 M urea and separated in urea-sucrose gradients [4] and by isoelectric focusing (IEF). Preparative IEF in urea-sucrose gradients produced SVDV protein prove to be pure by the criteria of SDS-PAGE [4]. Isoelectric points of VP1, VP2 and VP3 were 8.3, 6.0 and 5.2, respectively. Urea-sucrose gradient clearly sepa-

Fig. 1. Separation of SVDV proteins with SDS-PAGE. Full particles (F) or empty particles (E) were isolated with sucrose gradient, disrupted in SDS, and electrophoresed on SDS-PAGE. Lanes show the Coomassie blue staining of isolated viral proteins. Solid arrows indicate protein isolated from SVDV. Numbers indicate molecular weight markers; e.g. 30K represent molecular weight 30,000. Lane F and E show the protein isolated from peak of full and empty particles respectively.

rated virus proteins into 2 fractions; one was VP2 and another was the mixture of VP1 and VP3. Each fraction was inoculated to guinea pigs to produce specific antisera with the same procedure as mentioned above.

The antisera obtained were tested for monospecificity with Western blotting as described in detail elsewhere [9]. In Lanes a, b, c of Fig. 2, guinea pigs were immunized with SDS-PAGE-derived viral proteins. Antibody to VP1 reacted to VP1 band of full particles, while same antibody reacted to VP1 and cross-reacted to VP0 of empty particles (Lane b). Antibody to VP2 reacted to VP2 and cross-reacted to VP0 of full particles and empty particles (Lane c). In Lane d, e, f of Fig. 2, guinea pigs were immunized with urea-sucrose gradient separated viral proteins. Antibody to the mixture of VP1 and VP3 reacted

mainly to VP1 in full particles (Lane e). This suggested that VP1 was more immunogenic than VP3. Antibody to VP2 reacted to VP2 and cross-reacted to VP3 of full particles in Western blotting (Lane f). To determine the antigenic nature of viral proteins the guinea pig antisera was tested for neutralizing and ELISA antibodies.

Assays for neutralizing antibody activity in guinea pig sera were tested by the 50% virus plaque reduction method. Equal volumes of virus (100 PFU/ml) and serum dilution were mixed, incubated for 60 min at 37°C and 0.2ml of the mixture was added to IB-RS-2 cell monolayers in duplicate. The cultures were incubated at room temperature for 60 min for the virus attachment, overlaid with 5 ml of growth medium containing 3% calf serum and 1% Difco Noble agar, and incubated at 37°C for 2 days under 5% CO₂-air mixture for the plaque development. The plaques were made visible by staining with 0.05% methylene blue in 10% formaldehyde solution and antibody titer was determined as reciprocal of the highest serum dilution at which the number of plaques was reduced by 50%.

The enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere [2]. Polystyrene microtiter plates were coated with purified SVDV (1 µg/ml) diluted in 0.05 M carbonate buffer (pH 9.6). Plates were incubated overnight at 6°C. Prior to use for the assay, the virus-coated plates were washed three times with washing buffer (PBS containing 0.05% Tween 20). Antiviral protein guinea pig serum, serially diluted in PBS, was added for 60 min incubation at room temperature. Then the wells were washed three times and the amount of antibody bound to antigen was measured by adding an excess of horseradish peroxidase-conjugated anti-guinea pig immunoglobulin (60 min at room temperature) and orthophenylenediamine as substrate. The enzyme-substrate reaction was stopped by addition of 8 N H₂SO₄ and the yellow-brown color produced was measured at 490 nm spectrophotometrically. Antibody titer to viral protein was expressed as reciprocal of the highest dilution of serum yielding a sample/control ratio of 2.0 or greater.

As seen in Table 1, with the antisera of guinea pigs immunized with SDS-PAGE-derived proteins, only antibody to VP2 protein neutralized the virus, while antibody to VP1, VP2 or VP3

Fig. 2. Reactivity of immunized guinea pig sera with viral proteins. Full particles (F) or empty particles (E) were electrophoresed with SDS-PAGE (Lane a to f), and blotted on nitrocellulose membranes. The membranes were tested by the immunoperoxidase method with antisera of guinea pigs immunized with, whole particles (Lane a), VP1 (b), VP2 (c), VP1, VP2 and VP3 (d), VP1 and VP3 (e), VP2 (f), respectively. Viral proteins as immunogen for Lane b and c were isolated with SDS-PAGE, and were injected to guinea pigs. Proteins as immunogen for Lane d, e and f were isolated with urea-sucrose gradient.

Table 1. Comparison of neutralization and ELISA antibody titers of guinea pig antisera prepared against SVDV particles and viral proteins

Immunogen	Reciprocal of antibody titer ^{a)}	
	Neutralization ^{b)}	ELISA ^{c)}
SDS-PAGE isolated		
VP1	<1	5.2
VP2	1.7	4.6
VP3	<1	5.2
Whole particles	5.9	4.6
Urea-sucrose gradient isolated		
VP1 and VP3	4.8	4.5
VP2	2.2	4.2
Whole proteins	3.9	4.6

a) Reciprocal of antibody titer was shown in log₁₀ units.

b) Reciprocal of antibody titer was determined with 50% plaque reduction assay.

c) Purified virus particles were used as antigen.

bound to whole particle antigens in ELISA. Antibody to VP1 or VP3 did not neutralize SVDV. On the other hand, with antisera of guinea pigs immunized with urea-sucrose gradient isolated viral protein, antibody to the mixture of VP1 and VP3 neutralized SVDV. Antibody to VP2 also neutralized SVDV. In ELISA, SVDV was bound with antibody to VP2 or to the mixture of VP1 and VP3.

Present results showed that VP2 as well as the mixture of VP1 and VP3 could induce the neutralizing antibody in guinea pigs. When VP1 or VP3 was isolated from SVDV in SDS-PAGE, it did not induce neutralizing antibody. When VP1 and VP3 were separated in urea-sucrose gradient as the mixture, it did induce neutralizing antibody. Due to the denaturing effects of dissociating agents, the proteins obtained would show different degree of denaturation, which depended on variation of isolation technique [4]. Ongoing experiments showed that naturally SVDV-infected convalescent swine sera reacted with VP1 using Western blotting method. Full particles were purified by sucrose gradient (15–45%), which were mentioned above. Isolated particles were disrupted in SDS, electrophoresed with SDS-PAGE, and blotted on nitrocellulose membrane. The membranes were tested by the immunoperoxidase method with convalescent swine sera. Only VP1 reacted to convalescent sera in Western blotting (unpublished observation). These observation suggested that VP1, as well as VP2, proteins were responsible to induce the neutralizing antibody for SVDV.

These results may have some implication for understanding of the neutralization process

among picornaviruses, which VP1, VP2 and VP3 would be responsible for neutralization in three different functions as shown in poliovirus [4]. And they could also influence the projects designed to obtain the synthetic vaccine, which was based on the concept that a small fragment of protein was responsible for the induction of neutralizing antibody [1], and the studies to see whether single gene or multiple genes products are responsible for induction of neutralizing antibody in picornaviruses.

ACKNOWLEDGEMENTS. We are grateful to Mr. Michio Itho and Mr. Yoshimichi Andoh for their nice photography.

REFERENCES

1. Bachrach, H. L., Moore, D. M., McKercher, P. D. and Polatnik, J. 1975. *J. Immunol.* 115: 1636–1641.
2. Beatrice, S. T., Katze, M. G., Zajak, B. A., and Crowell, R. L. 1980. *Virology* 104: 426–438.
3. Brown, F., Talbot, P., and Burrows, R. 1973. *Nature* 245: 315–316.
4. Dernick, R., Heukeshoven, J., and Hilbrig, M. 1983. *Virology* 130: 243–246.
5. Donaldson, A. I., Ferris, N. P., Knowles, N. J., and Barnett, I.T.R. 1983. *Res. vet. Sci.* 35: 295–300.
6. Jacobsen, M. F., and Baltimore, D. 1968. *Proc. Natl. Acad. Sci. U.S.A.* 61: 77–89.
7. Harris, T.J.R., and Brown, F. 1975. *Nature*, 258: 758–760.
8. Tokui, T., Kono, M., Tokuda, G., Kumagai, T., and Sasahara, J. 1975. *Natl. Inst. Anim. Health. Q. (Jpn.)* 15: 165–173.
9. Towbin, H., Staehelin, T., and Gordon, J. 1979. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350–4354.

要 約

豚水胞病ウイルス構造たんぱく VP1 および VP2 による中和抗体の産生 (短報)：津田知幸・徳井忠史・小野寺節 (農林水産省家畜衛生試験場)——豚水胞病ウイルス自然感染豚中和抗体は VP1 と反応したが、モルモットでは SDS-PAGE により分離された VP2, 尿素蔗糖密度勾配によって得られた VP1・VP3 混合物および VP2 によって中和抗体が誘導された。VP1, VP2, VP3 それぞれを注射されたモルモットでは ELISA によりウイルスに対する抗体産生がみられた。