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COMMON AND DIFFERENT ANTIGENIC PROPERTIES OF THE RABIES VIRUS GLYCOPROTEIN OF STRAINS SAD-VNUKOVO AND PITMAN-MOORE

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Summary. – Two fixed rabies virus strains, SAD-Vnukovo and Pitman-Moore (PM) were used as combined immunogens for the generation of hybridomas secreting specific monoclonal antibodies (MoAbs). The obtained hybridomas were primarily screened by an ELISA for production of MoAbs to antigen of SAD-Vnukovo strain. Six positive clones were established. A panel of MoAbs has been characterized according to reactivity in immunofluorescence, immunoblot, ELISA and neutralization tests. All MoAbs were positive in immunofluorescence when cells infected with the SAD-Vnukovo strain were used. By immunoblot, four MoAbs showed specificity for the viral glycoprotein of both SAD-Vnukovo and PM rabies strains. This pattern of reactivity indicated the existence of shared conformation-independent epitopes located on the related antigens. However, in ELISA, the tested MoAbs did not recognize viral glycoproteins of the PM strain. This indicates, that the different strain-specific conformations of the native glycoprotein determine the accessibility of the common linear determinants for respective antibodies. Only one antibody, with conformation-dependent glycoprotein specificity, was capable to neutralize the CVS strain of rabies virus.

Key words: rabies virus glycoprotein; antigenicity; monoclonal antibodies

Introduction

Rabies virus, the etiological agent which causes fatal diseases of the central nervous system, is of major importance in human and veterinary medicine. In this Rhabdovirus of the genus Lyssavirus, five structural proteins were identified: the large protein (L), the glycoprotein (G), the nucleoprotein (N), the nominal phosphoprotein (NS), and the membrane protein (M).

The G protein is the only protein component on the external surface of the virus particle. It is the major antigen responsible for the induction of virus neutralizing antibodies and immunity against lethal rabies infection (Crick and Brown, 1969; Wiktor *et al.*, 1973). However, MoAbs have revealed a great diversity of the antigenic structure of the G protein among rabies viruses (Flamand *et al.*, 1980; Dietzschold *et al.*, 1988).

This article describes the preparation of murine MoAbs specific for the envelope G protein of different strains of rabies virus and indicates the pattern of their reactivity. Our findings indicate, that the strain-specific conformation of this viral glycoprotein might be responsible for the distinct reactivity of MoAbs directed to the common antigenic structures.

Materials and Methods

Immunogens. The SAD (Street Alabama Dufferin)-Vnukovo strain of rabies virus (obtained from Prof. Selimov, Institute of Poliomyelitis and Viral Encephalitides, Moscow) was propagated in BHK-21 cells. Cells grown in minimal essential medium with 5 % bovine serum were infected with a virus-dose of 0.001 LD_{50}/cell, and the culture supernatant was harvested 4–5 days post infection.

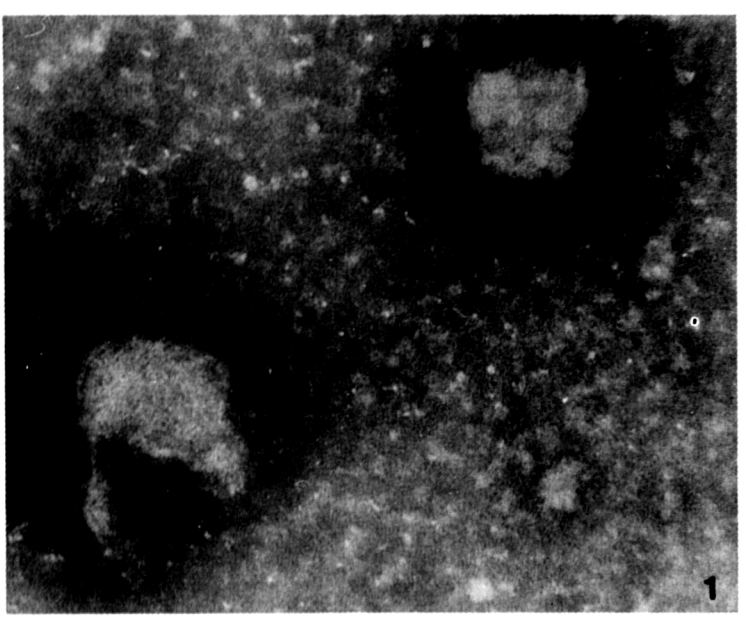

Fig. 1
Electron microscopy of negatively stained preparation of SAD-Vnukovo strain of rabies virus Magn. x 72 500.

The virus was inactivated with beta-propiolactone and the cell debris were removed by centrifugation at 10 000 x g/20 min. Purified virions were obtained as follows: Virus was pelleted at 20 000 rpm for 1.5 hr in the Beckman 45 Ti rotor, resuspended in a small volume of phosphate buffered saline (PBS) and layered onto sucrose gradient (20–60 % w/w in PBS). After 17 hr centrifugation at 50 000 rpm in the Beckman SW 55.1 rotor, the virus band was collected, adjusted with PBS to the protein concentration 1.2 mg/ml and stored at −70 °C. The presence of viral particles in the sample was confirmed by electron microscopy (Fig. 1).

The glycoprotein of the PM strain of rabies virus (Wistar rabies PM/WI38-1503-3M) was obtained as a commercially available rabies vaccine (lot A 0985, Institute Merieux, Lyon). The concentration of proteins in this vaccine was 1.5 mg/ml. The CVS strain of rabies virus was used in virus neutralization tests.

Electron microscopy. After attachment (2 min) to the formvar and carbon coated membrane grids the virus particles were stained with 2 % phosphowolframic acid (pH 7.0) for 1 min and investigated in the electron microscope Philips EM 300 at 80 kV.

Immunization. Groups of 4–6 week-old BALB/c female mice were injected intraperitoneally (i. p.) with 200 μg/mouse of purified virions of SAD-Vnukovo strain, emulsified 1:1 in the complete Freund's adjuvant (Sigma). Two months later, the mice were reinjected i. p. with 200 μg of antigen of PM strain in incomplete Freund's adjuvant. After four weeks, mice were boosted intravenously (i. v.) with 200 μg of PM strain antigen, dissolved in PBS. Three days later the mice were sacrified, their spleens removed and used for hybridoma production.

Hybridomas were generated by fusion of immune spleen cells with NSO mouse myeloma cells, as described elsewhere (Kontseková *et al.*, 1988). Rabbies virus-specific antibody-secreting hybridomas were screened by ELISA using viral antigen of SAD-Vnukovo strain. Selected hybridomas were cloned in soft agar and cultivated in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10 % horse serum. Hybridoma culture supernatants, or, ascitic fluids were used in experiments. Ascitic fluids were obtained from BALB/c mice primed i. p. with 0.5 ml paraffine oil and inoculated 5 days later with $5x10^6$ hybridoma cells. Subclass determination and light chain composition analysis of the MoAbs were performed using subclass-specific and light chain-specific anti-mouse immunoglobulin (Ig) antisera (Miles) by the Ouchterlony double diffusion technique.

ELISA. 96-wells microtitre plates (Labsystems) were coated overnight at 37° C with rabies virus antigen (10 μg/ml in PBS, pH 7.2; 50 μl/well). Plates were washed with 0.03 % Tween 20 in PBS (PBS-T) and the residual protein binding sites were blocked with 1 % nonfat dried milk for 1 hr at room temperature. Plates were washed with PBS-T and 50 μl aliquots of the hybridoma culture supernatant were added into wells for 1 ht at 37 °C. Then the plates were washed with PBS-T and 50 μl of peroxidase-conjugated rabbit anti-mouse Ig (DAKO) was added per well for 50 min at 37 °C. After washing, o-phenylenediamine substrate solution was added and the reaction was stopped after 20 min by the addition of 2 mol/l sulphuric acid. The plates were read at 492 nm using the ELISA reader Multiscan MCC/340 (Labsystems). Colour intensities at least twofold of the control were considered positive.

The specificity of the anti-rabies MoAbs for different epitopes was determined by the ELISA-additive assay (Solomon *et al.*, 1989). Plates were coated with antigen of SAD-Vnukovo rabies strain as described above. The intensities of the reaction of MoAbs assayed individually and in combination were compared. For the additive assay, 50 μl of hybridoma supernatants, either singly or in pairs, were added per well. When pairs of MoAbs were assayed, 50 μl of 1:1 supernatant mixtures were tested. The plates were then processed as described above for ELISA.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Rabies virus antigen (50–100 μg) was boiled 5 min in 100 μl of sample buffer with 1 % SDS. The electrophoresis of virus proteins was carried out in a 5–20 % polyacrylamide gel system (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Blue and vacuum-dried.

Immunoblot analysis. Proteins resolved by electrophoresis in SDS-PAGE were electroblotted to a polyvinylidene difluoride membrane (0.45 μm, Immobilon, Millipore). The electrotransfer of separated proteins was carried out for 1 hr at 148 mA either in 20 mmol/l Tris plus 150 mmol/l glycine, pH 8.3 (Tris-buffer) or in 10 mmol/l CAPS, pH 12 (CAPS-buffer). The blot was incubated for 1 hr with the blocking solution (1 % nonfat milk in PBS), and 3 mm wide strips were cut from the

blot and incubated overnight at 4 °C with single hybridoma culture supernatants. After 3 washes with PBS-T, the strips were treated for 1 hr at room temperature with anti-mouse Ig peroxidase conjugate. After 5 washes with PBS-T, diamino benzidine was added as substrate and the reaction was stopped by washing the strips with tap water.

Indirect immunofluorescence test. The BHK-21 cells growing on Terasaki plates were infected with SAD-Vnukovo strain of rabies virus and fixed 48 hr later with 80 % acetone in distilled water at room temperature. Acetone-fixed preparations were stored at -20 °C. To test hybridoma supernatants, the frozen plates were thawed and washed twice with PBS. Culture supernatants were added in 10 μl aliquots per well for 30 min at 37 °C. After washing anti-mouse Ig fluorescein isothyiocyanate conjugate (Institute of Sera and Vaccines, Prague) was added for 30 min at 37 °C. Washed and dried plates were examined using an epifluorescence microscope (Leitz).

Virus neutralization test. Serial twofold dilutions of ascitic fluid were mixed with an equal volume of the CVS strain of the standard challenge virus at concentration 50 LD_{50}/ml. The test mixtures were incubated for 1 hr at 37° C and then injected intracerebrally (i. c.) into BALB/c mice of 10–15 g weight (0.03 ml per mouse).

Results

In the immunization schedule, two rabies virus strains, SAD-Vnukovo and PM were combined to elicit antibodies against the common determinants of both immunogens. The virions of SAD-Vnukovo strain were used both for primary immunization and for screening of hybridomas. The viral antigen of PM strain was applied as the second immunizing dose and was injected into mice as the booster before fusion. Fourteen hybridomas were selected, which secreted MoAbs with binding ability to viral antigen of SAD-Vnukovo strain, as detected by primary ELISA-screening. Out of them six clones producing specific antibodies with the highest titer were chosen for the further study (Table 1). They were tested by indirect immunofluorescence for reaction with the viral antigen

Table 1. Hybridoma clones producing monoclonal antibodies to rabies virus

No.	Clone	Ig- Isotype	SAD-Vnukovo supernatant	ELISA titer[a] ascites
1	Rab 3	G 1 . k	10^{-3} - 10^{-4}	10^{-6}
2	Rab 18	G 1 . k	10^{-3}	10^{-5}
3	Rab 25	G 1 . k	10^{-2}	10^{-3}
4	Rab 44	G 1 . k	10^{-3}	10^{-4}
5	Rab 50	G 2b. k	10^{-4}	10^{-5}
6	Rab 52	G 1 . k	10^{-2} - 10^{-3}	10^{-4}
	N 54[b]	G 1 . k	< 1	< 10^{-2}
	NSO[c]	-	< 1	< 10^{-2}

[a] SAD-Vnukovo strain of rabies virus used as antigen. Titer corresponded to the highest antibody dilution which gave a twofold reading of the NSO-control.

[b] Clone producing irrelevant MoAb to interferon alpha 2.

[c] Ig- nonsecreting myeloma cells.

expressed on the acetone-fixed BHK-cells infected with SAD-Vnukovo strain. All six MoAbs were found positive in this assay (Table 2). To define the specificity of prepared antibodies, hybridoma culture supernatants were further analysed by direct binding to the structural viral proteins of SAD-Vnukovo strain in an immunoblot assay. The four structural proteins, i. e. G, N, NS and M protein, were visualised after SDS-PAGE (Fig. 2, lane b). When resolved proteins were electroblotted in Tris-buffer, only the antibody Rab 44 showed reactivity with a protein band adjacent to the glycoprotein (results not shown). However, when the same electrotransfer was repeated in CAPS-buffer, three others MoAbs (Rab 3, Rab 18, Rab 25) were also able to bind to the viral G-protein (Fig. 3 lane b). The additive-ELISA experiments with four MoAbs (Rab 3, Rab 18, Rab 25, Rab 44) were carried out to determine the number of epitopes which they recognized (Fig. 3). The obtained results indicate that the antibodies were directed at least to two different antigenic structures localized on rabies virus glycoprotein of SAD-Vnukovo strain. MoAbs Rab 3 and Rab 18

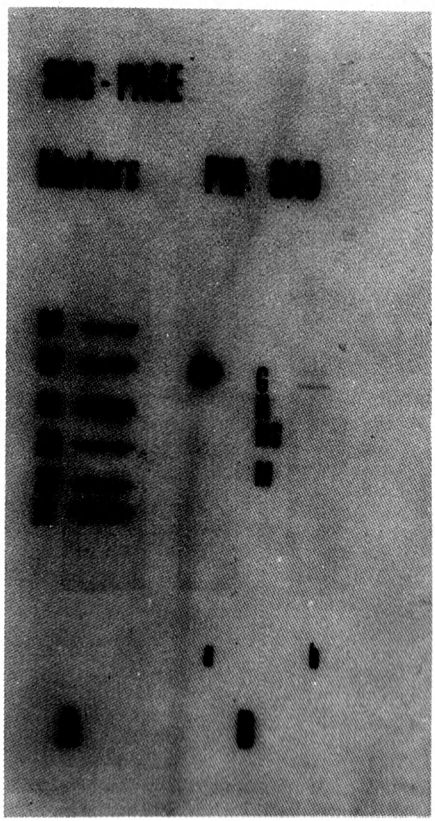

Fig. 2
SDS-PAGE of rabies viral antigens
Molecular weight markers (MW); Pitman-Moore strain (a); SAD-Vnukovo strain (b).

probably recognize the same or overlapping sites located on one antigenic sructure, while MoAbs Rab 25 and Rab 44 were directed to overlapping epitopes located on another antigenic structure.

The MoAb-panel was then tested for reactivity with the second immunogen, i. e. the PM strain of rabies vaccine. The SDS-PAGE of this preparation showed the presence of only one protein band, corresponding by its molecular weight to the viral glycoprotein (Fig. 2, lane a). In immunoblot (CAPS-buffer), the protein was recognized by the same four antibodies, which showed specificity for the G protein of SAD-Vnukovo strain, i. e. MoAbs Rab 3, Rab 18, Rab 25 and Rab 44 (Fig. 3, lane a). This cross-reactivity indicates the existence of shared conformation-independent epitopes located on the glycoprotein of both tested rabies virus strains.

However, ELISA experiments showed that all six MoAbs with binding capacity to the antigen of the SAD-Vnukovo strain failed to recognize the

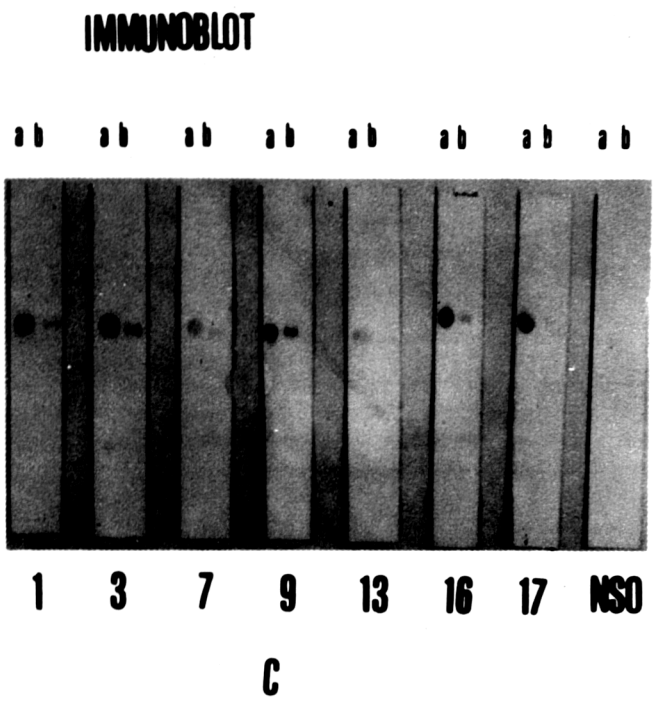

Fig. 3
Immunoblot analysis by monoclonal antibodies
Reactivity of indicated supernatants with antigen of PM strain (a) and SAD-Vnukovo strain (b). CAPS-buffer used for the electroblotting.

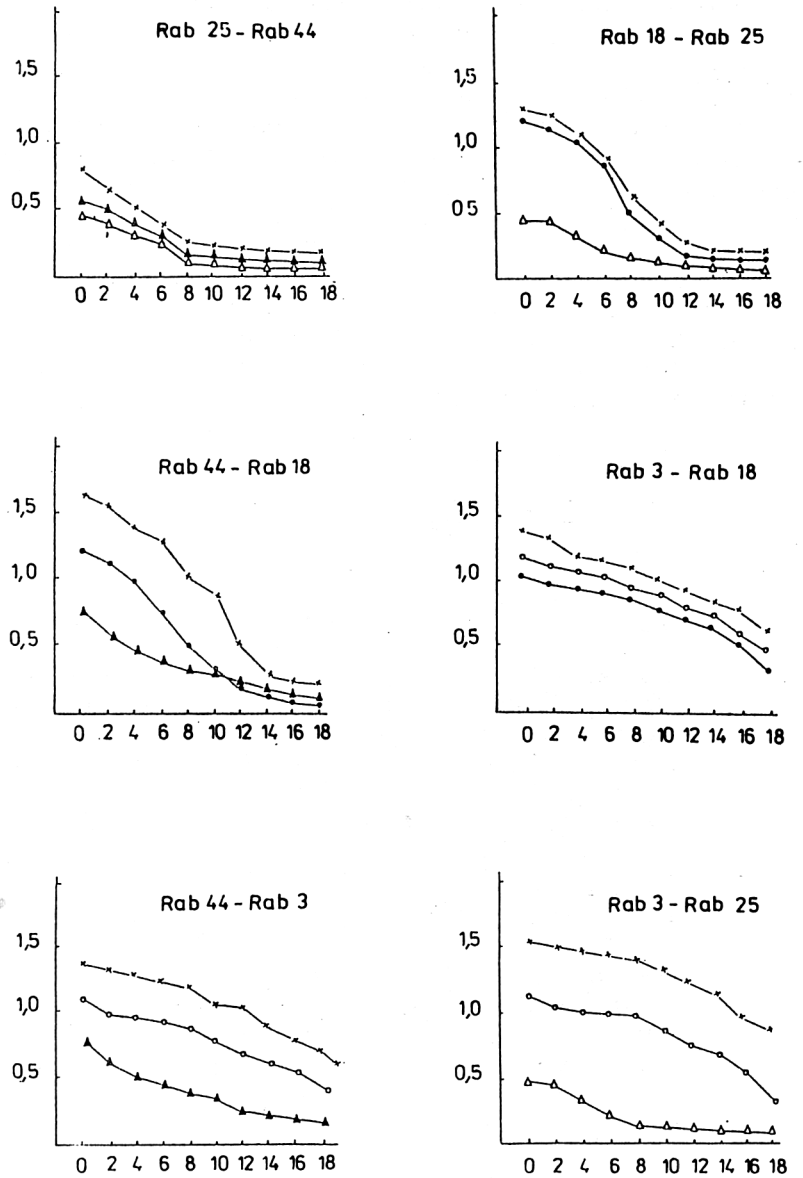

Fig. 4
ELISA-additivity test
MoAb Rab 3 (○), MoAb Rab 18 (●), MoAb Rab 25 (△), MoAb Rab 44 (▲), supernatants tested in pairs (x). Ordinate: A_{492}. Abscissa: dilution of hybridoma supernatant in log 2.

Table 2. Reactivity of monoclonal antibodies in immunofluorescence (IF), immunoblot, ELISA, and virus-neutralization test (NT)

MoAb	IF	Immunoblot		ELISA		NT
	SAD-Vnukovo	SAD-Vnukovo	PM	SAD-Vnukovo	PM	CVS
Rab 3	+	+	+	2.3	0.13	ND
Rab 18	+	+	+	1.8	0.16	-
Rab 25	+	+	+	1.7	0.10	-
Rab 44	+	+	+	1.5	0.08	-
Rab 50	+	-	-	2.1	0.16	+
Rab 52	+	-	-	1.1	0.06	-
N 54[a]	-	-	-	0.15	0.03	ND
NSO[b]	-	-	-	0.12	0.07	ND

[a] Irrelevant MoAb to interferon alpha 2.

[b] Ig- nonsecreting myeloma cells used as control.
ND - not done

glycoprotein of the PM strain (Table 2). These findings indicate the lack of close antigenic relationship between the native glycoproteins of compared rabies virus strains.

All MoAbs except Rab 3 were also characterized according to their activity to neutralize the CVS strain of rabies virus (Table 2). Surprisingly, only one antibody (Rab 50), that did not react in immunoblot had virus-neutralizing capacity. Thus the specificity of the MoAb Rab 50 for the viral envelope glycoprotein was demonstrated (Cox *et al.*, 1977; Wiktor *et al.*, 1984).

Discussion

There is a great diversity in the antigenic structure of the glycoprotein among different strains of rabies viruses (Flamand *et al.*, 1980; Dietzschold *et al.*, 1988). To address this problem, we tried to elicit the glycoprotein-specific MoAbs directed to common antigenic determinants of SAD-Vnukovo and PM rabies strains. To achieve this goal, both viral immunogens (complete virions of SAD-Vnukovo strain and glycoprotein of PM strain) were used in combination for immunization. MoAbs were primarily screened by ELISA for reactivity with virions of rabies virus of SAD-Vnukovo strain. From six MoAbs used in this study, five were unambigously shown to be raised against the rabies virus glycoprotein, while the MoAb Rab 52 was positive only in IF (SAD-Vnukovo strain). This high frequency of glycoprotein-specific antibodies might result from the use of purified envelope glycoprotein as the booster before fusion.

In the immunoblot analysis, four MoAbs reacted with the viral glycoprotein of SAD-Vnukovo strain. The G-specific antibodies recognized at least two diffe

rent epitopes on the antigen of SAD-Vnukovo strain. All four MoAbs reacted also with the viral glycoprotein of PM strain, suggesting close antigenic relationship between SAD-Vnukovo and PM virus strains. The pattern of MoAb-reactivity in the immunoblot analysis suggested a conformationally less dependent character of shared epitopes. These findings were quite surprising, because with exception of the report of Bunschoten *et al.* (1989), all previously identified epitopes on the rabies virus glycoprotein proved sensitive to denaturation after SDS-PAGE treatment (Dietzshold *et al.*, 1982; Wunner *et al.*, 1985; Vincent *et al.*, 1988). From the methodical aspect it is interesting, that the different reactivity of MoAbs in the immunoblot analysis, seems to depend on the electrotransfer protocol.

The proposed antigenic relationship between the virus strains SAD-Vnukovo and PM, as revealed by the immunoblot analysis, appears to be in contradiction to conclusions based on experimental data provided by ELISA. The ELISA data did not support the antigenic relation between the two rabies virus strains. To explain this discrepancy we propose different conformation-dependent accessibility of respective MoAbs to common linear epitopes located on the viral G protein of SAD-Vnukovo and PM strains. We suppose that the native conformation of glycoprotein of SAD-Vnukovo strain allowed both the access and the binding of single MoAbs to specific epitopes, while that of the PM strain could block the antibodies from reacting with the buried common sequential antigenic determinants. The proposed strain-specific exposition of the common linear epitopes on the native antigen is supported by the lack of reactivity of MoAbs with radiolabelled G protein of PM strain (data not shown). When this glycoprotein was injected as immunogen into mouse organism, a conformational alteration could make its common epitopes accessible for the immune system.

Out of five antibodies tested, only the MoAb Rab 50 had neutralizing activity against the CVS virus strain. The lack of binding ability of this antibody in the immunoblot analysis was in agreement with observations that virus-neutralizing antibodies preferentially recognize conformational epitopes (Dietzshold *et al.*, 1982; Wummer *et al.*, 1985; Vincent *et al.*, 1988). The cross-neutralization of the CVS strain by MoAb generated against the PM virus strain was also reported by Bunschoten *et al.* (1989). The remaining three antibodies tested failed to neutralize the CVS strain in spite that they were G protein-specific. However, MoAbs which reacted with the viral glycoprotein in the immunoblot analysis, but were not able to neutralize the virus have also been demonstrated (Bunschoten *et al.*, 1989).

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