Studies on the different conditions for rabies virus neutralization by monoclonal antibodies #1-46-12 and #7-1-9

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Virus-neutralizing activity of two monoclonal antibodies (mAbs), #7-1-9 and #1-46-12, against rabies virus glycoprotein (G) was compared. Although these mAbs affected the virion's ability to bind to host cells similarly, a big difference was found in the titres of virus neutralization (1:7132 and 1:32 for mAbs #1-46-12 and #7-1-9, respectively, at a concentration of 10 µg protein/ml). Although no big difference in virion-binding affinity between the two mAbs was found, the number of antibodies required for virus neutralization was very low, ≤ 20 molecules for mAb #1-46-12 and ≥ 250 molecules for mAb #7-1-9. In the latter case, the mAbs cover a major part of the virion surface and cause steric hindrance of viral receptor-binding activity. The infectivity of an epitope-preserved escape mutant virus (R-61) was not affected by the binding of high numbers of mAb #1-46-12 to the virion, which implies that mAb binding does not mask the receptor-binding site of the viral spikes. Based on these results, it is hypothesized that mAb #1-46-12 affected virus infectivity by a mechanism different from covering the virion spikes. Possible virus-neutralizing mechanisms by low numbers of mAb #1-46-12 in comparison to that of mAb #7-1-9 are discussed.

Introduction

The glycoprotein (G) of rabies virus has been studied extensively for many years because of its importance in the antigenicity for protective immunity and involvement in determining the neurovirulent nature of the virus (reviewed by Kawai & Morimoto, 1994; Coll, 1995).

Immunization with rabies virus vaccine usually results in the induction of protective immunity against rabies virus infection in the vaccinee: inactivated virion vaccines have been used for protecting humans (post-exposure treatment) and domestic animals (prophylactic use). In this case, although cellular immunity is not established (Hirai et al., 1992; Perrin et al., 1996), induction of antibodies against the G protein is known to work effectively for protection (Wiktor et al., 1973). Raised antibodies are thought to be involved in some antiviral activities in the body, such as virus neutralization by direct antibody binding to the virions or the indirect effects through complement activation (antibody-dependent complement-mediated virus lysis) and host cell killing by either antibody-dependent cell-mediated cytotoxicity or antibody-dependent complement-mediated cytotoxicity (Lafon, 1994; Thraenhart et al., 1994).

Prophylactic administration of antibody to a victim bitten by a rabid dog may be one of the effective measures for post-exposure treatment, for which anti-G monoclonal antibodies (mAbs) of known nature might be better than polyclonal antibodies (pAbs) for producing more effective results. Accordingly, more profound studies on the nature and properties of the neutralizing epitope are necessary for each mAb to be selected for its practical uses in post-exposure treatment of dog-bitten victims (Rando & Notkins, 1994).

Extensive studies have been done for two decades for anti-G mAbs, such as: fine structure studies; mapping the epitopes involved in virus neutralization; epidemiological and evolutionary studies on diversity and epitope-negative mutations of the G protein; and the use of mAbs for post-exposure treatment (Flamand et al., 1980; Coulon et al., 1982, 1983; Dietzschold et al., 1983, 1988, 1990; Lafon et al., 1983; Seif et al., 1985; Prehaud et al., 1988; Flamand et al., 1993).

We have prepared many stocks of rabies virus-neutralizing mAbs, among which only one mAb (#7-1-9) recognizes a linear epitope that has been previously studied precisely (Ni et
al., 1995). Since mAb #7-1-9 has the lowest specific virus-neutralizing titre, we performed further studies on other anti-G mAbs that were shown to be greatly different in virus-neutralizing activities. Among these, mAb #1-46-12 showed the strongest virus-neutralizing activity and was shown also to recognize a highly conserved conformational epitope; escape mutants that were resistant to this mAb were shown to have a specific amino acid substitution either at Thr\(^{16}\) or at Ser\(^{29}\) (Irie et al., 2002).

In this study, we compared the virus-neutralizing activities of anti-G mAbs, especially those mAbs having very weak (mAb #7-1-9) and very strong (mAb #1-46-12) virus-neutralizing activities. From our experimental studies on these mAbs (e.g. affinity of binding to the virion, effect on the virus attachment to the host cells, estimation of the minimum numbers of antibodies required for neutralization, etc.), we assume that binding of only a low number of mAb #1-46-12 to the virion was enough for virus neutralization, while mAb #7-1-9 required much higher numbers of antibody molecules, probably to cover the entire surface of the virion. Along this consideration, we hypothesize further that these two mAbs neutralize virus infectivity by different mechanisms.

**Methods**

**Rabies virus strains and cell cultures.** Rabies viruses used in this study were the laboratory BHK-adapted strains (HEP-Flury, ERA and CVS) (Kawai, 1977; Ni et al., 1995) and a vaccine strain (Nishigahara) (Sakamoto et al., 1994). All virus strains were plaque-purified and propagated once in BHK-21 cells. Virus stocks were titrated by plaque formation assays using BHK-21 cells, as described before (Kawai & Takeuchi, 1992). BHK-21 cells were propagated in Eagle’s minimum essential medium supplemented with 5% calf serum and 10% tryptose phosphate broth (Difco). In some studies, a mouse neuroblastoma cell line (C1300, subclone NA) (Morimoto et al., 1992) was also used.

**Preparation of escape mutants.** Preparation of escape mutants was described in our previous study (Irie et al., 2002). In brief, a working stock of the rabies virus HEP-Flury strain was passaged six times in the presence of mAb #1-46-12 (5 µg/ml). The mAb-resistant viruses were plaque-cloned and propagated in BHK-21 cell cultures. One of the 1-46-12 epitope-preserving isolates, termed R-61, was used for this study. The glycoprotein (G) of R-61 virus was shown to have an amino acid substitution at Ser\(^{29}\) with Thr, which might have allowed the G protein to have an additional N-glycan at Asn\(^{37}\) whereby the mutant virus could survive in the presence of mAb #1-46-12 (Irie et al., 2002).

**Preparation and purification of radiolabelled rabies virus.** Rabies virus-infected BHK-21 cells were metabolically labelled 40 h after infection with \[^{35}S\]methionine (10 µCi/ml or 3.7 × 10\(^{7}\) Bq/ml) for 6 h. Culture fluids were harvested and clarified by high-speed centrifugation at 10 000 g for 15 min. The supernatant was then centrifuged at 35 000–40 000 r.p.m. for 90 min through a 20% sucrose cushion. The pellet was suspended in PBS for neutralization experiments with mAbs.

**pAbs and mAbs.** Rabbit pAbs against the rabies virus G and N proteins were the same as those described in our previous study (Nakahara et al., 1999; Kawai et al., 1997). Preparation of mouse hybridoma clones was already described in our previous report (Ni et al., 1995). Viral G protein-specific hybridoma clones were screened by immunoprecipitation and immunoblot assays. Most of our G protein-specific hybridoma clones produced conformational epitope-specific mAbs, except for one (mAb #7-1-9), which produced a linear epitope-specific mAb (Ni et al., 1995). In this study, culture fluids of the hybridoma cells were mostly used.

**Virus neutralization assay by focus formation inhibition (FFI).** Virus-neutralizing activity of antibody solutions was determined by a modified method of the FFI assay described by Louie et al. (1975). In brief, virus solutions of wild-type (wt) and mutant viruses were diluted to give a concentration of 8 × 10\(^{4}\) p.f.u./ml (to give a final m.o.i. of 0.01 p.f.u. per cell when inoculated to BHK-21 cells at 1 × 10\(^6\) cells per dish), to which the same volume of serial 2-fold dilutions of mAbs (highest concentration was 10 µg protein/ml) was added. Reaction mixtures were incubated for 1 h at 37 °C, followed by inoculation to BHK-21 cells grown on glass coverslips. After 24 h of incubation at 36 °C, infected cells were fixed with acetone and fluorescent antibody (FA)-stained using anti-N antibody. Stained specimens were subjected to counting the numbers of FA-positive cell clusters (foci) in 20 fields under an epifluorescence light microscope.

**Host cell-binding test.** Radiolabelled virions were mixed with various concentrations of mAbs and incubated for 1 h at 37 °C. Then, each reaction mixture was inoculated to BHK-21 cell monolayers and incubated for 1 h at 4 °C for virus attachment to host cells. After washing the dishes three times with PBS, host cell-bound virions were recovered from the dishes by dissolving them in SDS–PAGE sample buffer and dot-blotted onto filter papers. After being dried, the radioactivity of the samples was analysed using a Bio-Imaging Analyser BAS2000 (Fuji Photo Film) to estimate the number of host cell-bound virions per sample.

**SDS–PAGE.** Assay samples were dissolved in sample lysis buffer (125 mM Tris–HCl, 4.6% SDS, 10% 2-mercaptoethanol, 0.005% bromophenol blue and 20% glycerol; pH 6.8). Samples were then applied to 10% polyacrylamide gels prepared with Laemmli’s discontinuous buffer. After electrophoresis, protein bands were stained with Coomassie brilliant blue R-250 (Merck).

**Semi-quantitative immunoblot assay.** The concentration of mAbs in culture media as well as the numbers of mAbs bound to the virions were estimated by a semi-quantitative immunoblot assay using a mouse mAb stock (#3-9-16) of known concentration (1 mg protein/ml) as a standard immunoglobulin (lg) solution as follows: a series of 2-fold dilutions (2\(^{-}^{1}\)–2\(^{-}^{7}\)) of the test mAb sample and a series of 4-fold dilutions (4\(^{-}^{1}\)–4\(^{-}^{5}\)) of the standard mouse lg sample were applied to the same gel slab of 15 lanes. Samples were lysed in sample lysis buffer and analysed by SDS–PAGE on a 10% polyacrylamide gel. After electrophoresis, the proteins in the gel were blotted electrodically onto a nitrocellulose filter (type BA85) (Schleicher & Schuell) using a semidry-type blotting apparatus, followed by blocking with skimmed milk. The filters were incubated with rabbit peroxidase-conjugated antibody against the murine immunoglobulins. After colour development, the density of colour in each band was compared to find the dilution of test samples showing similar colour densities to that of the standard sample. Using this method, stocks of hybridoma cultures were estimated to contain about 10 µg protein/ml of antibodies.

**Estimation of the amount of mAb bound to each virion.** Purified virion preparations (about 1–2 µg protein per tube) were mixed with various concentrations of mAb dilutions (starting from 10 µg/ml, serially diluted 2-fold). After incubation for 1 h at 37 °C, each mixture was divided into two parts, one of which was subjected to an FFI assay to determine residual virus infectivity and the other used to estimate the
number of mAbs bound to the virions by semiquantitative, luminescent immunoblot assays. The latter was done as follows: reaction mixtures were subjected to ultracentrifugation to recover the virion-bound mAbs by placing them on a 20% sucrose cushion in NTE buffer (140 mM NaCl, 50 mM Tris—HCl and 5 mM EDTA; pH 7.4) and centrifuged for 90 min at 35,000 r.p.m. After centrifugation, the supernatant was first removed and the centrifuge tube was filled again with PBS to wash the inside surface of the tube. The virion pellet was dissolved in 100 µl of SDS–PAGE sample buffer, serially 2-fold diluted with sample buffer and applied to two sets of SDS–PAGE gels. After SDS–PAGE, the proteins in the gels were processed for immunoblot assays as described above. After incubation with rabbit anti-N antibody and then with peroxidase-conjugated second antibody (goat) or with peroxidase-conjugated antimurine IgG antibody (goat), filters were processed for luminescence assays using a commercial luminescence assay kit (Western Blotting Luminol reagent, SC-2048; Santa Cruz Biotechnology) (Barroso & Santisteban, 1999). Luminescence emitted by Luminol oxidation was measured using an application soft NIH Image 1.6.2.

Results

General properties and virus-neutralizing activity of anti-G mAbs

One of our anti-G mAb stocks (mAb #1-46-12) was shown to recognize a highly conserved conformational epitope of the G protein (Irie et al., 2002). Another neutralizing mAb (mAb #7-1-9) was reported to recognize a linear epitope that was mapped to a middle region of the primary structure of the G protein and substitution of Phe263 with Leu completely abolished antigenicity (Ni et al., 1995).

These two mAbs showed a big difference in virus-neutralizing activity at the same antibody concentrations; the neutralizing titre of mAb #1-46-12 (10 µg protein/ml) was 1:7132 against the HEP-Flury strain, while that of mAb #7-1-9 was very low at 1:32 (Fig. 1). Such a high virus-neutralizing activity of mAb #1-46-12 was also displayed against other strains of rabies virus, including the CVS, ERA and Nishigahara strains (Fig. 2). On the other hand, mAb #7-1-9 similarly displayed a very low titre of virus-neutralizing activity against the CVS and ERA strains (data not shown), while the Nishigahara strain lacked the 7-1-9 epitope due to the replacement of the amino acid at position 263 by Leu (Ni et al., 1995). Based on these basic data, we defined further the difference of the two mAbs, especially the difference in their virus-neutralizing activity.

Virus replication step that is affected by the mAbs

We first examined whether the neutralized virions have lost their ability of attaching to the host cell. Radiolabelled virions were exposed to various concentrations of the mAbs (the starting concentration of 5 µg protein/ml was enough to inactivate virus infectivity). Antibody-treated virions were tested for their residual infectivity and the ability to attach to the host cell. Residual infectivity was determined by inoculating the samples to BHK-21 cell monolayers at a 1000-fold dilution and processing as described for the FFI assay. For testing host cell-binding ability, antibody-treated virions were inoculated to BHK-21 cell monolayers without any dilution, followed by processing for detection of the cell-bound virions, as described in Methods.

![Fig. 1. Comparison of virus-neutralizing activity of the mAbs against rabies virus G protein. Diluted virus solutions (about 10⁵ p.f.u./ml) of rabies virus (HEP-Flury strain) were mixed with the same volume (0.2 ml) of a series of 2-fold dilutions of each hybridoma culture fluid (original concentration of mAb was about 10 µg protein/ml). After incubation for 1 h at 37 °C, residual infectivity was checked by inoculating the mixtures (0.2 ml) to BHK-21 cells sown on glass cover slips (10⁶ cells per dish). Infected cultures were incubated for 24 h at 36 °C and fixed with acetone for 15 min at room temperature, followed by FA staining with rabbit antibody against the rabies virus N protein and then with FITC-conjugated second antibody. The number of fluorescence-positive foci of infected cells was determined for each sample under an epifluorescence light microscope. Reciprocal of the ratio of foci number (n) to that of infected control (n₀) [i.e. % inhibition = (1 – n/n₀) x 100] was calculated and plotted against concentrations of each mAb. –, mAb #1-46-12; –, mAb #4-11-42; –, mAb #4-7-15; –, mAb #4-45-33; –, mAb #4-50-42; –, mAb #7-1-9.

![Fig. 2. Similar virus-neutralizing activity of mAb #1-46-12 against different laboratory strains of rabies virus. Virus-neutralizing activity of mAb #1-46-12 was examined against several laboratory strains (i.e. CVS, ERA, HEP-Flury and Nishigahara strains) of rabies virus in the same way as that described in Fig. 1. Results obtained were treated similarly for being plotted against the mAb concentrations. –, HEP-Flury; –, CVS; –, ERA; –, Nishigahara strains.](image-url)
Inhibition of virus infectivity and attachment in parallel by two mAbs $g_1$-46-12 and $g_7$-1-9. (A) Radiolabelled virions (10^8 p.f.u./ml) were mixed with the same volume (0–2 ml) of a series of 2-fold dilutions of mAbs (original concentration was about 10 µg protein/ml). After incubation for 1 h at 37°C, reaction mixtures were divided into two parts, one of which was subjected to examination of cell-binding ability of the virion, as described in Methods. Ratio of the amount of bound virions and that of original virions applied to the dish was calculated for each reaction mixture. The other half was used for determination of the residual infectivity by FFI assay (see Methods). Reciprocals (i.e. % inhibition) of the ratio of the numbers to those of infected control was calculated and plotted against the concentrations of mAbs. E and _, neutralization of virus infectivity by mAbs $g_1$-46-12 and $g_7$-1-9, respectively; D and ^, inhibition of virion attachment by mAbs $g_1$-46-12 and $g_7$-1-9, respectively. (B) The data shown in (A) were plotted again to show a linear relationship between the inhibition of virus infectivity and that of host cell attachment in relation to the antibody concentration. E, mAb $g_1$-46-12; ^, mAb $g_7$-1-9.

As shown in Fig. 3, each mAb affected in parallel the infectivity and the host cell-binding ability of the virions, although the antibody concentration for 50% inhibition was quite different for each mAb. These data indicate that the neutralized rabies virions lost their ability to attach to the BHK-21 cell. Similar results were also obtained when a mouse neuroblastoma cell (a subclone NA of C-1300) was used as the host cell for the assay.

**Kinetic studies on mAb binding to the virion**

We performed time-course studies on mAb-binding to the rabies virions. As shown in Fig. 4, two mAbs displayed similar virion-binding kinetics. This observation implies that there is no big difference in the affinity to antigens of the two mAbs to explain the big difference in virus-neutralizing titres.

To confirm this assumption, we examined further a possible correlation between the antibody concentration and the amounts of virion-bound antibodies. Fig. 5 shows that the two mAbs behaved similarly to give similar virion-binding kinetics, that is similar numbers of mAbs are bound to the virions at the different concentrations.

**Fig. 3.** Inhibition of virus infectivity and attachment in parallel by two mAbs #1-46-12 and #7-1-9. (A) Radiolabelled virions (10^8 p.f.u./ml) were mixed with the same volume (0–2 ml) of a series of 2-fold dilutions of mAbs (original concentration was about 10 µg protein/ml). After incubation for 1 h at 37°C, reaction mixtures were divided into two parts, one of which was subjected to examination of cell-binding ability of the virion, as described in Methods. Ratio of the amount of bound virions and that of original virions applied to the dish was calculated for each reaction mixture. The other half was used for determination of the residual infectivity by FFI assay (see Methods). Reciprocals (i.e. % inhibition) of the ratio of the numbers to those of infected control was calculated and plotted against the concentrations of mAbs. E and _, neutralization of virus infectivity by mAbs #1-46-12 and #7-1-9, respectively; O and △, inhibition of virion attachment by mAbs #1-46-12 and #7-1-9, respectively. (B) The data shown in (A) were plotted again to show a linear relationship between the inhibition of virus infectivity and that of host cell attachment in relation to the antibody concentration. E, mAb #1-46-12; △, mAb #7-1-9.

**Fig. 4.** Time-course study on the interactions between anti-G mAbs and rabies virions. Radiolabelled rabies virions (about 10 µg protein) were mixed with the same volume (0–2 ml) of Parsorbin cells (formalin-fixed Staphylococcus aureus ghost cell suspension; Calbiochem), which had been coated in advance with test mAbs (10 µg IgG/0–2 ml), followed by incubation for various periods (20, 40, 60 and 120 min) at 37°C. The Parsorbin cell-bound virions were recovered by centrifugation at 5000 r.p.m., washed with PBS and processed for determination of the radioactivities, which were plotted against incubation periods; E, mAb #1-46-12; △, mAb #7-1-9.

**Fig. 5.** Comparisons of antibody binding kinetics between two anti-G mAbs. The wt and R-61 virions (about 5 µg protein/ml) were mixed with the same volume (0–2 ml) of serially 3-fold diluted mAb solutions (concentration of the original sample was 5 µg protein/ml) and incubated for 1 h at 37°C. Then, the antibody-bound virions were recovered by ultracentrifugation and applied to two sets of SDS–PAGE gels, followed by semi-quantitative immunoblot assays using the peroxidase-conjugated anti-murine Ig antibody and the rabbit anti-rabies N antibody and peroxidase-conjugated anti-rabbit second antibody; E and _ and O and △, mAb #1-46-12 bound to the wt and R-61 mutant virions, respectively; △ and △, mAb #7-1-9 bound to the wt and R-61 mutant virions, respectively. Dashed and dotted lines depicted in this figure indicate the maximum numbers of #1-46-12 and #7-1-9 antibody molecules bound per virion, respectively, as detected in the equilibrium phase of the incubation (6 h at 37°C).
same antibody concentrations in the 1 h incubation time. Based on these data, similar Scatchard curves were drawn for both the mAbs, indicating that both mAbs similarly displayed a high affinity of binding constant (K) of about $6 \times 10^9$ M$^{-1}$. In separate binding assay experiments, 707 and 770 lgG molecules per virion of mAb #1-46-12 and #7-1-9, respectively, were shown to be bound to the virion at the equilibrium phase (i.e. 6 h incubation) of the binding assay (data not shown) [these lgG molecule numbers per virion seem to be roughly half of the total numbers (about 1350) of the virion G protein reported for the rabies virus by Dietzschold et al. (1979)].

Minimum numbers of bound mAbs required for virus neutralization

We expected a certain relationship between the numbers of virion-bound mAbs and the loss of virus infectivity. Accordingly, we tried to estimate the minimum number of each mAb required for neutralization of virus infectivity. For this purpose, numbers of virion-bound mAbs were estimated by semi-quantitative immunoblot assays using a mouse mAb sample of known concentration as a standard (see Methods).

Purified virions (about 2 µg protein/ml) were mixed with various concentrations of the mAbs and incubated for 1 h at 37 °C. Then, the virion-bound mAbs were recovered by ultracentrifugation and subjected to SDS–PAGE and immunoblot assays with peroxidase-conjugated anti-mouse IgG goat antibody and the antibody against rabies virus N protein. The numbers of virion-bound mAbs were estimated by comparing the developed colour densities of the heavy chain (molecular mass is 55 kDa) of test mAb with those of a standard IgG sample of different amounts. Then, each value was converted to the number (n) of antibody molecules per virion based on the content of the N protein (molecular mass is 54 kDa) for each sample as follows: $n = \frac{0.5 \times [\text{amount of heavy chain (µg)]/[\text{amount of N protein (µg)}] \times 1800 \times \text{lgG molecules bound per virion}]}{1800}$, where 1800 is the number of N protein molecules per virion (Kawai, 1977). As shown in Fig. 6, it was revealed that binding of only low numbers (20 or less) of antibody per virion was enough for neutralization by mAb #1-46-12 antibody (more than 300 antibody molecules per virion), while mAb #7-1-9 similarly affected both mutant and wt viruses by binding of high numbers of antibody molecules to the G protein spikes of the virion (Fig. 6).

For the last experiment, we performed mixed infection studies to investigate whether the mutant phenotype of R-61 is dominant, especially concerning the sensitivity to neutralization by mAb #1-46-12 when the virion spikes are composed of both wt and mutant G proteins. BHK-21 cells were co-infected with the wt and R-61 mutant viruses at the same m.o.i. of 3 p.f.u. per cell. Progeny virions were recovered from the culture at 48 h after infection and examined for their sensitivity to mAb #1-46-12.

As shown in Fig. 7(A), progeny virions of the mixed infection were as highly sensitive to mAb #1-46-12 as the wt virions, although the virions contained the G proteins of both wt and R-61 viruses (Fig. 7B). This result suggests that mixed infection produced mosaic virions containing the G proteins of both wt and mutant viruses but such mosaic virions did not show the resistant nature against the mAb as the R-61 virion did.

Discussion

In this study, we compared two anti-rabies mAbs (#1-46-12 and #7-1-9) to characterize their different virus-neutralizing activity. They showed very similar binding affinities to the viral G protein antigen but the difference was found in the minimum numbers of bound antibody molecules per virion that were required for neutralization of virus infectivity.

As to the mechanisms of virus neutralization by antibodies, some possible ways have been proposed for many viruses. A simple mechanism of virus neutralization is to block virus attachment to host cells by masking the receptor-binding site of virion surface protein (steric hindrance theory). mAb #7-1-9 would inhibit rabies virus infection in this way, since the minimum requirement of mAb binding was about 200–250 antibody molecules per virion, while total numbers of virion spikes have been estimated to be about 445 per virion, indicating that mAb coverage of about 60% of the virion spikes would be necessary for steric hindrance of virus attachment to the host cells. The steric inhibition mechanism of virus neutralization by an anti-rabies mAb was also described by Flamand et al. (1993). Burton et al. (2001) reviewed a similar mechanism of virus neutralization for other viruses. Average numbers of bound IgG required for 63% (i.e. 1–1/e) neutralization were in proportion to the surface area of the virion and were calculated by a formula: numbers (N) of IgG required for neutralization = $0.0033 \times A$, where A is the surface area of the virion. Examples of the calculated N value for phage MS2, poliovirus, human rhinovirus, papillomavirus, influenza A virus and rabies virus were 4, 4–5, 6–7, 38, 70 and 225, respectively (Burton et al., 2001). On the other hand, requirement of mAb
For legend see facing page.
### Table 1. Comparisons of G protein between the wt (HEP-Flury) and its escape mutant R-61

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<td>Sensitivity to mAb</td>
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<td>Potential N-glycosylation sites*</td>
<td>37-NLS</td>
<td>319-NKT</td>
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* An original potential glycosylation site at position 158 was lost by amino acid substitution at Ser by Pro during serial passages of the virus through BHK-21 cell cultures (Irie et al., 2002).

Fig. 7. Neutralization of the mosaic virus prepared by co-infection with HEP-Flury wt and R-61 viruses by anti-G mAb 1-46-12. (A) The wt virus of HEP-Flury strain and R-61 mutant were mixed at a ratio of 1:1 and inoculated to BHK-21 cell cultures (m.o.i. of 3 p.f.u. per cell). After 48 h of incubation, progeny virions were examined for their sensitivity to mAb 1-46-12 at various antibody concentrations, as described in Fig. 1. For control studies, original wt and R-61 viruses were also checked in a similar manner as that for the test mosaic virus. ▲, Mosaic virus (progeny of wt + R-61 co-infection); ○, wt virus; ■, R-61 virus. (B) Virions of each virus sample (2 ml of infected culture fluid) were recovered by ultracentrifugation (35000 r.p.m. for 90 min) and subjected to two sets of SDS–PAGE, followed by immunoblot analysis with the rabbit anti-G (G) and anti-N (N) antibodies. Lanes: 1, wt; 2, R-61; 3, wt + R-61. G1, Glycosylated at one site; G2, glycosylated at two sites. (N) Prepared to ensure that similar amounts of progeny viruses were produced in the co-infected culture as that in the singly infected cultures.

#1-46-12 was very low in number, at 20 antibody molecules or less, which implies a possibility of different neutralization mechanisms other than steric hindrance of the virus–host cell interactions.

Virus neutralization by relatively small numbers of IgGs has been reported for some viruses. A low number of IgGs bound to the haemagglutinin of influenza A virus could inhibit viral transcriptase activity resulting in virus neutralization (Possee et al., 1982). Non-enveloped rhinovirus has been suggested to undergo molecular rearrangement of the capsid proteins, as revealed by an altered pl value of the proteins, by binding of one or two IgG molecules to the spikes: this is also thought to affect the host cell-attaching ability of the virus (Colonno et al., 1989). As for neutralization by mAb #1-46-12,
the neutralized virions lost host cell-attaching ability, being different from the case of viral transcription inhibition of influenza A virus by anti-HA antibody binding.

Flamand et al. (1993) described an epitope-positive escape mutant J12 of the rabies virus (CVS strain), which showed the same affinity to anti-G mAb 30AA5 as the wt virus did, although cell-attaching ability of the wt virus was not originally affected by the mAb but only the fusion activity was blocked. As to our epitope-preserving escape mutant R-61, we think that receptor-binding site of the spike of R-61 virus was not masked or blocked by binding of a high number of mAb #1-46-12 molecules. From this viewpoint, we could speculate further that mAb #1-46-12-mediated inhibition of host cell attachment of the wt virus would not be due to masking of the receptor-binding domain of the G protein.

We hypothesize that inhibition of virus attachment by mAb #1-46-12 is not due to the complete masking of all of the G protein spikes of the virion but that mAb binding to low numbers of the G protein spikes is enough for the neutralization. Antibody binding would induce conformational changes of the G protein with a kind of domino effect, which might finally cause abolishment of the receptor-binding ability of the virion. Concerning the neutralizing mechanism of mAb #1-46-12, we have to think about the effect of amino acid substitution at Ser59 by Thr and the additional N-glycosylation at Asn37 of the G protein on the spike structure of R-61 virus. As we reported previously (Anzai et al., 1997), one amino acid change at position 389 of the rabies virus N protein from Ser to analogous Thr greatly affected the protein, losing the phosphatase-sensitive epitope for mAb #5-2-26. From this viewpoint and from the fact that additional glycosylation occurs at Asn37 of the G protein, which is not originally glycosylated as long as Ser59 is preserved, we can suppose that amino acid substitution at Ser59 affects the structural rigidity or flexibility of the G protein, resulting in the acquisition of resistance to conformational changes possibly induced by binding of mAb #1-46-12. Alternatively, acquisition of the N-glycan at Asn37 did not affect the epitope structure and the mutant R-61 virions showed similar values of antibody binding. Accordingly, an additional N-glycan at Asn37 might hinder or block the mAb-induced conformational changes of the G protein. Either of these changes might also give a kind of potential distortion to the G protein that might be revealed when mutant G proteins are solubilized from the virion. This assumption seems to be supported by our preliminary experiments, in which epitope-positive mutant G proteins became incapable of being precipitated by mAb #1-46-12 when released by Nonidet P-40 or CHAPS from the virions (unpublished observation). This may be due to some conformational changes of the proteins occurring after being released from the virion. These points are now under investigation.

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