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Protection of Mice from Rabies by Intranasal Immunization with Inactivated Rabies Virus

Atsushi YONEDA[1)], Kotaro TUCHIYA[2)], Yasuhiro TAKASHIMA[3)], Takeshi ARAKAWA[4)], Naotoshi TSUJI[5)], Yoshihiro HAYASHI[1)], and Yasunobu MATSUMOTO[1)]

[1)]Department of Global Animal Resource Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, [2)]Nippon Institute for Biological Science, 2221–1 Shin-machi 9-chome, Ome, Tokyo 198-0024, [3)]Department of Veterinary Parasitological Diseases, Faculty of Applied Biological Sciences, Gifu University, 1–1 Yanagito, Gifu 501-1193, [4)]Division of Molecular Microbiology, Center of Molecular Biosciences, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, and [5)]Laboratory of Parasitic Diseases, National Institute of Animal Health, National Agricultural Research Organization, 3–1–5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

Abstract: *The mucosal immunization method is a needle-free alternative way of vaccination. This study evaluated the efficacy of mucosal immunization for rabies. Mice were intranasally administered five times with inactivated and concentrated rabies virus antigen (CRV) supplemented with or without cholera toxin (CT). The anti-rabies virus antibody titer of mice intranasally immunized with CRV plus CT (CRV/CT) was comparable to that of mice intraperitoneally immunized twice with the same amount of CRV. Virus neutralizing (VNA) titers of mice immunized intranasally with CRV/CT were slightly lower than those of intraperitoneally immunized mice. Both anti-rabies virus ELISA antibody and VNA titers of mice immunized with CRV without CT were significantly lower than those of mice immunized with CRV/CT. In mice intranasally immunized with CRV/CT, and intraperitoneally immunized mice, high levels of IgG_{2a} antibody were detected, suggesting the activation of Th1-driven cellular immunity by the two ways of immunization. All immunized mice were challenged intracerebrally with a lethal dose of virulent rabies virus CVS strain. The survival rates of mice immunized with CRV/CT and CRV without CT were 67% and 17%, respectively, while the rate of intraperitoneally immunized mice was 100%. Antigen-specific whole IgG and IgG_{2a}, and VNA titers of survived mice were significantly higher than those of dead mice at the challenge day. These data suggest the possibility of intranasal immunization with inactivated antigen as a rabies vaccination strategy and the importance of a mucosal adjuvant such as CT.*

Key words: *challenge infection, cholera toxin (CT), intranasal immunization, rabies virus*

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Address corresponding: Y. Matsumoto, Department of Global Animal Resource Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Introduction

Rabies is a viral zoonosis and carnivores are the main reservoir of the rabies virus. Globally, more than 2.5 billion people live in regions where rabies is endemic. It is estimated that, yearly, at least 500,000 people take post-exposure vaccinations and that 50,000 people die from rabies [20].

Nowadays, effective injectable vaccines against rabies are available for both humans and animals. However, injection of vaccines is painful with a risk of blood-borne infectious diseases when a single set of a needle and a syringe is used for two or more people. Furthermore, the cost of needles, syringe, vials and cooling equipment is quite high, as is the cost of discarding them safely. Oral rabies vaccines in which recombinant vaccinia virus-based vaccine or attenuated vaccines were used have been used for wild canine species [4, 5, 24, 32]. Since these oral vaccines are live vaccines, there is a risk of spreading in the field or of giving virulence by back- or cross-mutations.

Recently, mucosal immunization using recombinant proteins or inactivated pathogens has been studied for both mucosal [15, 19, 22, 25, 26, 28, 29, 30] and non-mucosal pathogens [1, 2, 16, 23]. Due to the ease of administration and the efficacy against both mucosal and non-mucosal pathogens, a mucosal immunization strategy with recombinant protein is considered as one of the most promising alternative vaccination methods.

In this study, we examined the potential of the safe mucosal immunization against rabies by immunizing mice intranasally with inactivated rabies virus.

Materials and Methods

Reagents

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Gamma chain specific), HRP-conjugated goat anti-mouse IgG_1, and HRP-conjugated goat anti-mouse IgG_{2a} were purchased (ZYMED, San Francisco, CA). HRP-conjugated goat anti-mouse IgA (SIGMA Aldrich Japan, Tokyo, Japan) was also used in this study.

Buffers

PBS buffer (1.4 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), TNE buffer (50 mM TrisHCl, 140 mM NaCl, 5 mM EDTA, pH 7.8), protein loading buffer (50 mM Tris-HCl. pH 6.8, 2% SDS, 840 mM beta-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue), and phosphate citrate buffer (103 mM Na_2HPO_4, 52 mM citric acid, pH 5.0) were used in this study.

Viruses and cells

Rabies virus CVS strain was propagated in ddY mouse brains and used for virus neutralization tests and challenge infections. Rabies virus RC-HL strain derived from the Nishigahara strain was propagated in HmLu cells. HmLu cells were grown in Eagle's minimum essential medium (E-MEM, Invitrogen, San Francisco, CA) supplemented with 10% heat-inactivated FCS and 10% tryptose phosphate broth (TPB, Difco Laboratories, Detroit, MI). As a source of an inactivated rabies virus antigen, Nisseiken rabies TC™ vaccine, which contains rabies virus RC-HL strain derived from the Nishigahara strain inactivated with β-propiolactone, was purchased from Nisseiken CO. (Nisseiken CO., Tokyo, Japan) and used as a source of the inactivated rabies virus antigen for immunization.

Mice

Six-week-old female BALB/c mice were purchased from Japan SLC (SLC, Shizuoka, Japan). Six-to-eight-week-old female ddY mice were purchased from Saitama Experimental Animals Supply CO., Saitama, Japan.

Monoclonal antibodies

Rabies virus glycoprotein (G protein)-specific monoclonal antibody, 2B9, was established by one of the authors, Dr. K. Tuchiya. Briefly, this hybridoma was prepared from splenocytes of BALB/c mice immunized with purified rabies virus RC-HL strain. Immunization of mice, cell fusion, selection and cloning of anti-G protein antibody-producing B cell hybridomas were performed according to the standard method [14]. Ascites from BALB/c mice injected with 5×10^6 2B9 hybridoma cells were used for detecting rabies virus G protein in western blot analysis.

Preparation of concentrated inactivated rabies virus (CRV)

Rabies virus particles were concentrated by ultracentrifugation for intranasal administration. Nisseiken rabies TC™ vaccine was overlaid on 45 w/v% and 10 w/v% sucrose in TNE buffer and centrifuged at 58,424 × *g* at 4°C for 90 min using SW28 rotor (Beckman, Tokyo, Japan). The visible virion layer between the 10 and 45% sucrose layers was collected, and dialyzed against TNE buffer at 4°C overnight.

SDS-PAGE, silver staining and western blot analysis

SDS-PAGE and western blot analysis were performed according to standard methods. Briefly, aliquots of the protein were boiled for 5 min in loading buffer, and subjected to electrophoresis in 10% polyacrylamide gels. Silver staining was performed using 2D-SILVER STAIN-II "DAIICHI" Kit (Daiichi Pure Chemicals CO., LTD., Tokyo, Japan). For western blot analysis, the proteins in the gel after separation were transferred to Immobilon polyvinyl transfer membrane (Millipore, San Francisco, CA). The membranes were blocked with 3% skimmed milk (S-PBS) at room temperature for 1 h, and were incubated with anti-rabies virus G protein monoclonal antibody 2B9 (1:5000). Following three washes with PBS, each wash being 5 min, membranes were treated with HRP-conjugated anti-mouse IgG antibody, at a 1:5000 dilution at 37°C for 1 h, and washed again three times. Then, positive signals were visualized by treating membranes with ECL western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) at room temperature for 1 min, followed by exposing the membrane to an X-Omat X-ray film (Kodak, Tokyo, Japan).

ELISA and determination of rabies virus-specific Ig ELISA titers

Sera from immunized mice were analyzed for the presence of rabies virus antigen-specific antibodies by ELISA. All wells in 96-well plates were coated with 5 μl of RV in 100 μl of 0.1 mM sodium carbonate, pH 9.5, and left overnight at 4°C. Then, after washing 3 times with 0.1% Triton X-100 in PBS (T-PBS), the wells were blocked by incubating with S-PBS at 37°C for 1 h. In order to diminish the reaction of serum antibodies against FCS components which were carried-over into TC vaccine solution in trace amounts, 1 μl of mouse sera was incubated with 100 μl of FCS in 1,024 μl of S-PBS at 37°C for 2 h. After incubation, serum samples were serially diluted twofold in S-PBS, applied to an ELISA plate and incubated at 37°C for 1 h. After washing 3 times with T-PBS, plates were incubated with HRP-conjugated antibodies against mouse IgG, IgG_1 and IgG_{2a} antibodies at 1:5000 dilutions in S-PBS, or against mouse IgA antibody at 1:3000 dilutions. After washing three times with T-PBS, 0.004% o-phenylanediamine and 0.003% H_2O_2 in phosphate-citrate buffer were added to each well and incubated at room temperature for 30 min in the dark. The reaction was terminated by adding 20 μl of 6 N H_2SO_4, and the absorption of each well at 490 nm was measured. Rabies virus-specific Ig titers were determined as a reciprocal of the highest serum dilution that showed an OD_{490} value above the cut-off value, which was defined as the average OD_{490} value of seven non immunized sera + 6 standard deviation.

Virus neutralization (VN) test

A rabies virus VN test was carried out on 96-well microplates as described previously [33]. Briefly, heat-inactivated serum samples were serially diluted twofold in E-MEM supplemented with 5% heat-inactivated FCS and 10% TPB. An equal volume of rabies virus RC-HL strain (2,000 $TCID_{50}$ / ml) was added to each serum dilution and incubated at 37°C for 2 h. After incubation, 100-μl aliquots of each mixture were put into three wells of 96-well plates and 50 μl of HmLu cell suspension at a concentration of 2×10^5 cell/ ml was added to these wells. The plates were incubated at 37°C for 6 days, and the appearance of cytopathic effect was examined under a light microscope. VN antibody (VNA) titer was determined as a reciprocal of the highest serum dilution that inhibited the cytopathic effect in two or three of the three wells.

Intranasal immunization and challenge infection

Mice were intranasally immunized five times with 30 μl of CRV in combination with 5 μg of cholera toxin (CT) adjuvant (CRV/CT) or without CT, on days 0, 14, 21, 28 and 35. Another group of mice which received intraperitoneal immunization was injected two times with CRV on days 21 and 35 of the intranasal immuniza-

tion schedule described above. This was done in order to innoculate these mice with rabies virus at the same time as the mice in the intranasally treated groups with five doses of rabies virus antigens. One week after the last immunization, mice were challenged intracerebrally with 12–50 LD_{50} of rabies virus CVS strain. Mice that died between the 5th and 14th days were considered to have died of rabies and were counted.

Results

As shown in Fig. 1A, the intensity of bands corresponding to rabies virus G, N, P and M proteins in CRV were higher than those in Nisseiken rabies TC™ vaccine, when the same amounts of virus solution, 10 μl, were loaded on SDS-PAGE, suggesting successful enrichment of rabies virus particles. On the other hand, the intensities of non-viral proteins, including those of 66 kDa and 29 kDa, were lower in CRV (Fig. 1A). Rabies virus soluble glycoprotein (Gs) of 50 kDa, shed from rabies virus particles [11] was also cleared off after ultracentrifugation (Fig. 1B). The band intensity of G protein in CRV serial dilutions was compared in western blots with those in Nisseiken rabies TC™ vaccine. The G protein band densities of CRV at 0.3 and 0.6 μl were similar to those of rabies virus in Nisseiken rabies TC™ vaccine at 5 and 10 μl, respectively, suggesting that rabies virus antigens in CRV were about 16-fold more concentrated than those in original Nisseiken rabies TC™ vaccine (Fig. 1B).

Groups of mice were intranasally immunized 5 times with a five-fold serial dilution of CRV, starting from 30 μl. One group of mice was intraperitoneally immunized twice with the same amount of CRV. One week after the last immunization, serum was collected from each mouse and the serum anti-rabies virus ELISA titer was measured. In mice intraperitoneally immunized with CRV, anti-rabies virus ELISA antibody titers of 30 μl-immunized mice and 6 μl-immunized mice were not different and much higher than those of 1.2 μl-immunized mice (Table 1). Rabies virus-specific antibody titers of mice intranasally immunized with 30 μl of CRV/CT were significantly higher (P<0.05) than those of mice receiving 6 μl of CRV/CT. Intranasal immunization with 1.2 μl of CRV/CT seemed much less effective (Table 1).

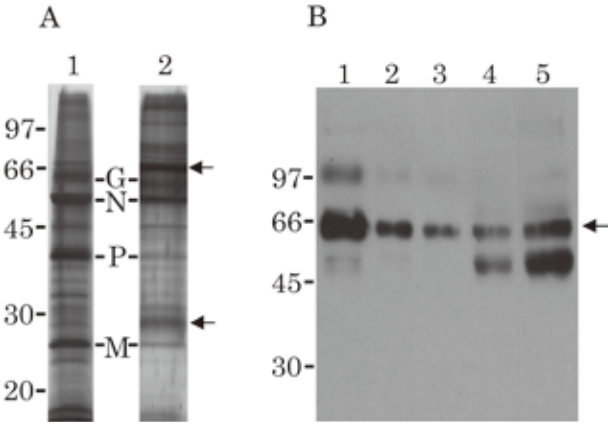

Fig. 1. Comparison of the amount of viral protein in concentrated RV (CRV) and original RV. (A) 10 μl of CRV purified by ultracentrifugation (lane 1) and original RV (lane 2) were subjected to SDS-PAGE and silver stained. Bands corresponding to the major viral proteins, G, N, P, and M, are marked. Non-viral 66 kDa and 29 kDa proteins were indicated by arrows. 220 kDa L protein was not clearly identified. (B) The amount of rabies G protein in CRV was compared with that in RV by western blot analysis using a G protein-specific monoclonal antibody. 1.2 μl (lane 1), 0.6 μl (lane 2), and 0.3 μl (lane 3) of CRV and 5.0 μl (lane 4) and 10 μl (lane 5) of RV were loaded. The G protein was indicated by an arrow. The band corresponding to the soluble glycoprotein (Gs) observed in lanes 4 and 5 disappeared after purification (lanes 1–3).

The anti-rabies virus IgG titer of mice intranasally immunized with 30 μl of CRV/CT (19.8 ± 1.0) was comparable to that of mice intraperitoneally immunized twice with 30 μl of CRV (19.7 ± 0.5), suggesting the possibility of raising rabies virus antigen-specific antibodies by intranasal immunization. Although anti-rabies virus IgG antibody was produced in mice which were intranasally administered CRV without CT, the levels of antibody titer were significantly lower than those of mice intranasally immunized with CRV/CT, suggesting the indispensable role of CT as a mucosal adjuvant. Serum IgA levels were also measured (Table 1). High levels of IgA were detected in mice immunized intranasally with CRV/CT. In mice immunized intraperitoneally with CRV, IgA levels were low, at a similar level with those in mice intranasally administered CRV without CT, though their IgG titers were significantly high.

The VNA titer of sera collected one week after the last immunization in groups of mice immunized with 30 μl of CRV was measured by the VN test as described in

Table 1. Rabies virus-specific IgG ELISA titers of mice immunized intranasally with CRV/CT (CT+) or CRV (CT–)

Vaccination route	Volume	IgG[a)]	IgA[a)]
Intranasal (CT+)	30 μl	19.8 ± 1.0	15.7 ± 0.8
	6 μl	18.2 ± 1.2	N.D.[b)]
	1.2 μl	14.0 ± 7.3	N.D.
Intranasal (CT–)	30 μl	15.5 ± 1.5	11.0 ± 1.5
	6 μl	15.5 ± 2.0	N.D.
	1.2 μl	9.2 ± 7.5	N.D.
Intraperitoneal	30 μl	19.7 ± 0.5	12.8 ± 0.8
	6 μl	19.0 ± 1.4	N.D.
	1.2 μl	12.2 ± 6.8	N.D.

[a)] Titers are expressed as geometric mean ± SD. [b)] Not determined.

Table 2. Rabies virus neutralizing antibody (VNA) titer and survival rate after challenge infection with a lethal dosage of rabies virus in mice immunized intranasally with CRV/CT (CT+) or CRV (CT–)

Vaccination route	VNA[a)]	Survival (rate)[b)]
Intranasal (CT+)	11.2 ± 1.9	4/6 (67%)
Intranasal (CT–)	8.7 ± 1.8	1/6 (17%)
Intraperitoneal	13.5 ± 1.0	6/6 (100%)

[a)] Titers are expressed as geometric mean ± SD. [b)] 7 days after the last vaccination, the mice were challenged intracerebrally with 38 LD_{50} of CVS strain.

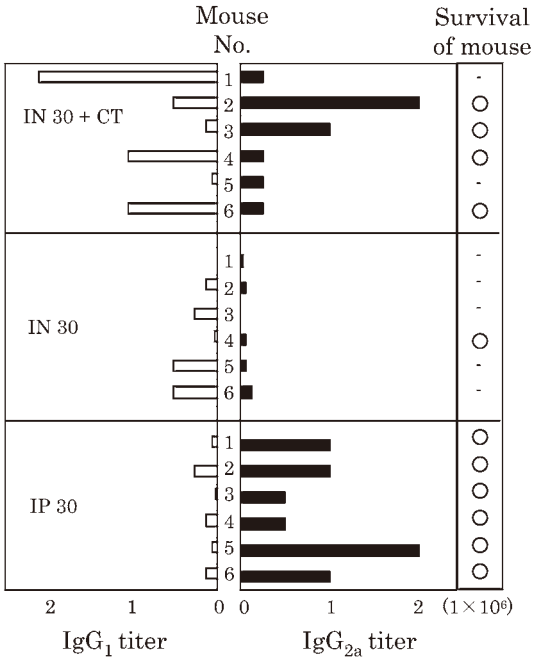

Fig. 2. IgG_1 and IgG_{2a} ELISA antibody titers of mice immunized intranasally and intraperitoneally with CRV, and their survival after rabies virus challenge infection. Rabies virus-specific IgG_1 (open bar) and IgG_{2a} (solid bar) antibody titers of each mouse are expressed as the geometric mean ± SD of the reciprocal of the highest dilution that showed an OD_{490} value above the cut-off value, determined as mean ± 6 SD of the OD_{490} value of 7 wells of pre-immunized sera in ELISA. (○) indicates survived mice and (-) indicates dead mice after challenge with a lethal dose of virulent rabies virus CVS strain.

Materials & Methods (Table 2). The VNA titer of mice immunized intranasally with CRV/CT was slightly lower than that of intraperitoneally immunized mice (11.2 ± 1.9 vs 13.5 ± 1.0). The VNA titer of mice immunized without CT (8.7 ± 1.8) was significantly lower than that of mice immunized with CRV/CT. Mice immunized with 30 μl CRV or CRV/CT were challenged with 45 LD_{50} of virulent rabies virus CVS strain one week after the last immunization (Table 2). All of the mice immunized intraperitoneally with CRV survived. Four out of six mice survived (67% survival) among mice intranasally immunized with CRV/CT, but the protective efficacy decreased dramatically (17%) when CT was omitted from the vaccine formulation.

Since both Th1- and Th2-type responses are known to be the important arms of protective immunity for rabies virus infection, serum anti-rabies virus IgG_1 and IgG_{2a} titers were measured in mice immunized intranasally or intraperitoneally with 30 μl CRV (Fig. 2). In all intraperitoneally immunized mice, we observed the level of IgG subclass was skewed to IgG_{2a}, suggesting the activation of Th1-derived cellular immunity. In mice administered intranasally with CRV without CT, both IgG_1 and IgG_{2a} levels were generally low, and more IgG_1 seemed to be induced than IgG_{2a}. However, intranasal immunization with CRV/CT, in the presence of CT, induced mixed IgG_{2a}/IgG_1. Interestingly, all mice intranasally immunized with CRV/CT showed high IgG_{2a} levels, whereas enhancement of IgG_1 production was only observed in some of them.

To evaluate the contribution levels of the types of humoral arms, anti-rabies virus IgG, IgG_1 and IgG_{2a} titers, as well as VNA titers, were compared in all survived

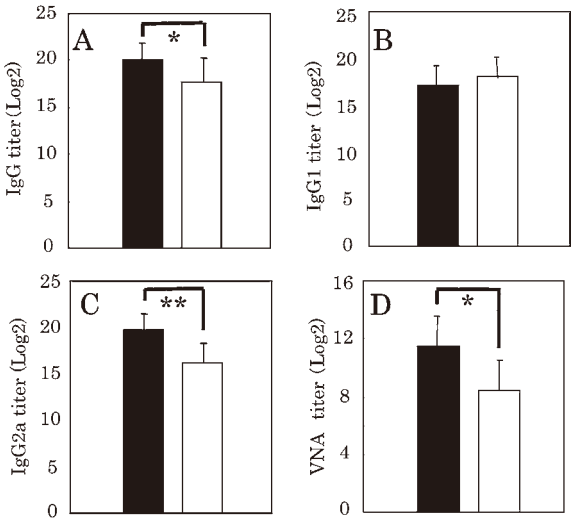

Fig. 3. Rabies virus-specific IgG, IgG_1, and IgG_{2a} ELISA titers, and VNA titers of survived and dead mice immunized with CRV by different methods, and challenged with virulent rabies virus. Rabies virus-specific IgG (A), IgG_1 (B), IgG_{2a} (C) ELISA titers and VNA titers (D) at one week after the last immunization of all survived (solid bar) and dead (open bar) mice immunized with 30 μl of CRV by different methods, and challenged with virulent rabies virus CVS strain. Titers are expressed as the geometric mean ± SD of the reciprocal of the highest dilution of each serum that showed a positive value. Statistical significance of difference was determined by the Student's *t*-test. *: $P<0.05$, **: $P<0.01$.

and dead mice immunized with 30 μl of CRV (Fig. 3). Titers of IgG, IgG_{2a} and VNA of survived mice (21.1 ± 1.7, 19.7 ± 1.7, 11.4 ± 2.1) were found to be significantly higher than those of dead mice (17.8 ± 2.4, 16.3 ± 2.1, 8.4 ± 2.1), however, no difference was observed in IgG_1 titers between survived and dead mice.

Discussion

The mucosal immunization method is a needle-free alternative way of vaccination. It is easier to vaccinate animals with the mucosal method than the parenteral method, and unlike parenteral vaccines, mucosal vaccination stimulates sIgA production in mucosal tissues, which could effectively inhibit the entry of pathogens that invade their hosts via the mucosal route. The efficacy of mucosal immunization has been well documented for mucosal pathogens, such as influenza virus, Newcastle disease virus, foot and mouth disease virus, Aujeszky's disease virus, HIV and *Ascaris suum* [15, 19, 22, 25, 26, 28, 29, 30]. Furthermore, mucosal immunization can also stimulate systemic immune responses, such as serum IgG production and cytokine production by T cells [28, 29]. Thus, mucosal vaccination can also be used for non-mucosal, penetrative pathogens, such as *Plasmodium* parasites, *Clostridium tetanus* and *Leishmania major* [1, 2, 16, 23]. In this study, the effective induction of protective immunity by intranasal immunization was also demonstrated in the parenteral pathogen, rabies virus. A mucosal immunization strategy capable of providing systemic as well as local protective immunity, and applicable to both mucosal and non-mucosal pathogens is considered one of the most promising alternative vaccination methods against infection.

For the practical application of a potential new rabies vaccine, five doses may not be acceptable, and many published studies have demonstrated successful intranasal immunization with less than three doses [1, 25, 26, 29]. In our study, mice intranasally administered 3 times with CRV/CT all died when intracerebrally infected with a lethal dose of virulent rabies virus, even though they showed some levels of anti-rabies virus ELISA antibodies and VNA (data not shown). In humans, the preventive vaccination for rabies is performed by three injections [7]. Thus, the efficiency of intranasal immunization for rabies has to be improved (e.g., by using more concentrated antigen, or a prime/boost strategy with a different administration route) [1, 13, 18].

Although rabies virus-specific ELISA antibody titers in mice immunized intranasally with CRV/CT, and those in mice immunized intraperitoneally with CRV, were comparable (Table 1), VNA titers were slightly lower in intranasally immunized mice than those in intraperitoneally immunized mice. The majority of VN antibodies to rabies virus have been shown to recognize structural epitopes at the site II region of G protein [3]. One of the reasons why VNA titers were lower in intranasal immunization may be due to the difference in antigen delivery and processing from mucosa to the body through M cells.

In the present study, survival from the lethal virus challenge significantly correlated with IgG_{2a} titers (Fig. 3). This suggests the importance of the activation of

cellular immunity. Dietzschold et al. demonstrated that immunization with internal N protein of rabies virus protects mice from rabies, suggesting that T cell activation is a major protective component in the immune system in rabies virus infection [10]. They also demonstrated that immunization with a T cell epitope enhanced rabies virus-specific antibody production [12]. Considering that antibody levels are low in the brain because of the blood-brain barrier [17], and that lymphocytes infiltrate relatively easily into the brain after virus infection, T cells may have an essential function in controlling rabies virus replication in the brain. CT is an effective mucosal adjuvant which stimulates both Th1 and Th2 types of immunity [8]. Compared with mice immunized intranasally with CRV, IgG_{2a} levels were much higher in mice immunized intranasally with CRV/CT (Fig. 2), suggesting that CT adjuvant enhanced antibody production and also Th1 activation.

Survival from challenge infection was also correlated with VNA titers (Fig. 3). Three surface molecules, the nicotinic acetylcholine receptors [6], neural cell adhesion molecule (NCAM/ CD56) [27], and the low-affinity nerve-growth factor receptor $p75^{NTR}$ [31] have been shown as receptors for the rabies virus. Since the VNA titers in this study were determined without complements, these neutralizing antibodies would have been able to inhibit the binding of the virus to these receptors, or the fusion process of the rabies virus as described by Dietzschold *et al*. [9]. Thus, it is reasonable to assume that VNA inhibits virus growth in the brain and that VNA titers correlate with the survival of immunized mice. Statistically, the survival of the immunized mice also correlated with the rabies virus-specific IgG ELISA antibody titers (Fig. 3). This might be the reflection of high VNA and IgG_{2a} titers in survived mice.

Edible vaccines are an excellent subject for mucosal immunization. Modelska *et al*. has demonstrated an edible vaccine for rabies by using a plant mosaic virus-based expression system with linear epitopes from G and N proteins of rabies virus [21]. However, the majority of neutralizing epitopes reside within the site II region of G protein, and many of them are conformational rather than linear epitopes. Thus, immunization with the full-length, properly folded G protein seems more desirable than immunization with a combination of several linear epitopes fused to a carrier molecule. Furthermore, when whole virus particles were used as antigens in the present study, even without CT adjuvant, antibody responses and VNA production were detected in intranasally immunized mice (Tables 1 and 2). When using recombinant protein as an antigen, it is rare to detect antibody production in intranasal immunization without CT or other adjuvants [2, 1, 30, 28]. Thus, the successful induction of anti-viral immunity demonstrated in our study by administration of CRV without CT may largely be attributed to the use of entire viral particles as immunogen. Similar results were also observed for Aujeszky's disease virus [26] and Japanese encephalitis virus (unpublished results). These results strongly support the hypothesis that viral proteins, e.g., G protein, gain stronger mucosal immunogenic property when attached to a larger carrier molecule, e.g., virion. The result that Gs protein, which is another form of G protein cleaved out from the surface of virions or infected cells, did not contribute to protection [11] also supports this hypothesis.

Although the efficiency of intranasal immunization needs to be improved, we clearly demonstrated in this study that a protective immune response could be elicited by mucosal vaccine in a murine model of rabies virus infection.

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