An immune stimulating complex (ISCOM) subunit rabies vaccine protects dogs and mice against street rabies challenge

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Dogs and mice were immunized with either a rabies glycoprotein subunit vaccine incorporated into an immune stimulating complex (ISCOM) or a commercial human diploid cell vaccine (HDCV) prepared from a Pitman Moore (PM) rabies vaccine strain. Pre-exposure vaccination of mice with two intraperitoneal (i.p.) doses of 360 ng ISCOM or 0.5 ml HDCV protected 95% (38/40) and 90% (36/40) of mice, respectively, against a lethal intracerebral (i.c.) dose with challenge virus strain (CVS). One 360 ng i.p. dose of ISCOM protected 87.5% (35/40) of mice against i.c. challenge with CVS. Three groups of five dogs were vaccinated intramuscularly (i.m.) with 730 ng of rabies ISCOM prepared from either the PM or the CVS rabies strains, and they resisted lethal street rabies challenge. Postexposure treatment of mice with three or four 120 ng i.m. doses of ISCOM protected 90% (27/30) and 94% (45/48), respectively, of mice inoculated in the footpad with street rabies virus, but three doses of HDCV conferred no protection. When four doses of HDCV were administered postexposure, 78% (32/41) of the mice died of anaphylactic shock; 21% (11/52) of mice had already died of rabies 4 days after the third vaccine dose was administered.

Keywords: Rabies; ISCOM; human diploid cell vaccine; postexposure; treatment; anaphylactic shock

INTRODUCTION

Rabies is endemic in most of the world, and rabid dogs are responsible for >90% of human rabies deaths[1,2]. Various types of human and animal vaccines are used worldwide for the prevention of rabies. These vaccines are prepared from brain tissue of adult or newborn animals[3–6], avian tissues[7–9], or cell cultures[10–12]. The virus strains used for vaccine production are usually derived from the Pasteur fixed rabies virus strain, and the virus is usually inactivated by physical or chemical methods[3–13]. In humans, rabies vaccines are used for both pre- and postexposure immunization, whereas in animals only pre-exposure vaccination is practised. Although the immunological basis of protection following vaccination with inactivated or live attenuated rabies virus vaccines is still not fully understood, it has been shown that both humoral and cellular immune responses are induced by rabies virus vaccines[14–16].

Rabies virus particles contain five different proteins. Closely associated with the nucleoprotein (N) are the non-structural (NS) protein and the virion transcriptase (L). The N-protein represents the group-specific antigen of the genus. The matrix (M) protein and the glycoprotein (G) are located in a lipoprotein envelope, through which spikes of G protein project[17–19]. Spike G-proteins are amphiphilic proteins with at least one subunit of the oligomeric protein inserted into the lipid bilayer of the viral envelope anchoring the spike to the membrane[20]. For rabies virus and most other enveloped viruses, the spike G-proteins are the antigens responsible for stimulating virus-neutralizing antibody[21–26]. It has also been shown that the N-protein (through another mechanism) can induce a protective immune response against rabies infection in animals[27,28].

One of the crucial points in eliciting immunity and protection is the effective presentation of the desired antigens in a vaccine. To date, relatively little attention

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has been paid to the physical form in which antigens should be presented to the immune system. Since it is now possible to isolate spike proteins as single peplomers from virion or through recombinant-DNA techniques, more immunogenic forms of presentation have become available such as protein micelles, virosomes, or the recently described immune stimulating complexes (ISCOMs). In addition, antigenically and physically defined subunit vaccines can now be formulated[15,16,29–33]. Although protein micelles or liposomes containing multiple copies of virus G-protein have been shown to have considerable immunogenicity[34,35], this immunogenicity largely depends on the addition of suitable adjuvant[36].

The ISCOM structure was developed to create a well defined submicroscopic particle, one that presents several copies of antigen exposed on a built-in saponin adjuvant (Quil-A)[35,37–39]. ISCOMs containing one or more viral envelope proteins of more than 20 different membrane viruses belonging to eight different virus families have been prepared; all were shown to be highly immunogenic in various animal species, and they induced high protective immunity and good T-cell response[37,40–44]. This report presents data on the serological response and protection from challenge in animals immunized with either an ISCOM containing the rabies G-protein or with a commercial human diploid cell rabies vaccine.

MATERIALS AND METHODS

ISCOM preparation

ISCOMS[35,45] were prepared from either the Pitman Moore (PM) or the challenge virus standard (CVS) rabies strains. Briefly, to ≈200 mg purified virus in 8 ml TN-buffer (0.05 M Tris–hydrochloride, pH 7.4, 0.1 M NaCl), 0.9 ml of a stock solution of detergent Mega-10 (decanoyl-*N*-methylglucamide) (Sigma)* was added to a final concentration of 2% and left for 2 h at room temperature. The solubilized virus was layered on top of 10% sucrose in TN buffer containing 0.2% Mega-10 (w/v), which was layered on 30% sucrose in TN buffer. The solubilized virus was concentrated at 30000 rev min^{-1} for 2 h at 20°C in a TST 41.14 rotor. The top fraction consisting of the sample volume plus the 10% sucrose layer was collected and Quil-A was added to a final concentration of 0.1%. The mixture was extensively dialysed for 72 h against three changes of TN buffer at room temperature.

For comparative purposes a commercial human diploid cell vaccine (HDCV) (Imovax, lot no. Z-1355) prepared from the PM strain, and a reference rabies vaccine prepared from CVS (kindly supplied by National Veterinary Laboratory, Iowa, USA) served as controls.

Purification of ISCOM by lectin affinity chromatography

Approximately 40 mg purified rabies virus was solubilized in 175 ml of 1% Triton X-100 solution and the viral G-protein was purified by affinity chromatography on a lentil lectin-conjugated Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden). The solubilized virus material was applied to the lectin column and equilibrated with TN-buffer containing 1% Triton X-100, and the unbound material was washed out of the column with 10 volumes of TN-buffer. Mega-10 was then introduced into the column at a concentration of 0.05% in TN buffer. Material specifically bound to the lectin column was eluted with 0.2 M methyl glycopyranoside dissolved in TN-buffer, containing 0.5% Mega-10. After elution peak, fractions were pooled and concentrated by a 30 K cut-off ultrafiltration device (Filtron) to a final volume of 25 ml. To this concentration 4 mg lipid was added from a stock solution containing 10 mg cholesterol and 10 mg phosphatidyl choline dissolved in water with 20% Mega-10. Quil-A was added to a final concentration of 0.1%, and the mixture was dialysed for 3 days against three changes of TN-buffer, pH 7.4, at room temperature. The formation of ISCOM was confirmed by determination of the S-value[46] in sucrose gradient centrifugation by negative contrast electron microscopy of the gradient fractions.

Animals

All animals used in this experiment were raised in closed colonies at the Centers for Disease Control animal breeding facility. None of the dogs had been previously vaccinated against rabies, and none had rabies virus-neutralizing antibodies (VNA) at the time of inoculation (vaccination).

Determination of antigenic values

The National Institutes of Health (NIH) potency test[47] was used to determine and standardize the antigenic values of the ISCOMs, HDCV and reference vaccine[48]. In brief, mice were vaccinated twice intraperitoneally (i.p.) at a one-week interval with fivefold dilutions of each antigen. The vaccinated mice, along with groups of unvaccinated control mice, were then challenged intracerebrally (i.c.) with 30–100 mouse intracerebral lethal dose 50% ($MICLD_{50}$) of CVS 1 week after the last vaccination. All test antigens were then diluted to obtain similar antigenic values for use in the protection experiments.

Determination of VNA titres

VNA titres in sera collected from experimental animals were determined by the rapid fluorescent focus inhibition test (REFIT)[49]. Results were expressed in international units (IU) using an international standard reference serum[49].

Immunofluorescence

All dog sera were also tested for anti-nucleocapsid antibody titre by the indirect fluorescent antibody (IFA) technique using fluorescein-conjugated rabbit anti-dog globulin.

Pre-exposure vaccination of mice

Groups of 4-week-old female CDs-ICR mice were vaccinated i.p. on days 0 and 7 with 0.5 ml containing 360 ng Gp of the respective ISCOM preparations, 0.5 ml (containing 2.5 IU) HDCV, or the reference vaccine prior to i.c. challenge with $10^{3.2}$ $MICLD_{50}$ CVS on day 14. All mice were observed for 30 days, and the number of dead animals was recorded daily.

* Use of trade name or commercial sources is for identification only and does not imply endorsement by the Public Health Services or the US Department of Health and Human Services

Postexposure vaccination of mice

To mimic the postexposure treatment of rabies infection, groups of mice were injected in the left hind footpad (FP) with 0.03 ml containing 100–200 $FPLD_{50}$ of street rabies (MD 5951) isolated from the salivary gland of a dog in Mexico. The mice were then vaccinated with 0.1 ml HDCV or 0.1 ml ISCOM preparation containing 120 ng once on day 0 (24 h postinjection (PI); three times (days 0, 3 and 7); or four times (days 0, 3, 7 and 14 PI). HDCV was given either three times (days 0, 3 and 7) or four times (days 0, 3, 7 and 14 PI). The animals were checked daily for clinical signs of rabies.

Pre-exposure vaccination of dogs

Twenty laboratory-raised beagles, 1 to 3 years of age and of either sex, were divided into four groups of five dogs each. Each dog was vaccinated i.m. in the hind leg. Group 1 was vaccinated with 1 ml containing 5 IU PM whole-virus suspension; groups 2, 3 and 4 were vaccinated with corresponding amounts (730 ng) of ISCOM antigen prepared from either the PM or the CVS strains. Serum specimens were collected at 30, 60 and 90 days for determination of VNA titres against CVS and street rabies virus strains. At the end of the 90-day observation period, all dogs, including five unvaccinated control beagle dogs, were challenged in the masseter muscle with 1 ml of canine salivary gland suspension containing $10^{6.3}$ ($MICLD_{50}$) of a street rabies virus obtained from a dog near the Texas–Mexico border (L-2596). Five days postchallenge, serum specimens were collected to determine the possible booster effect of the challenge virus.

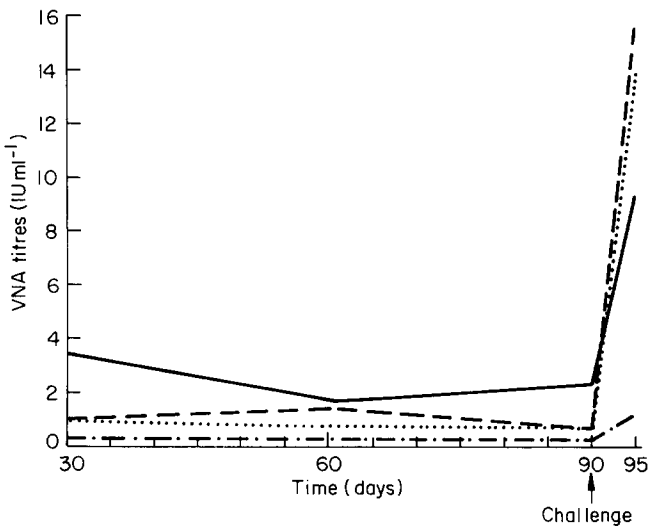

Figure 1 Geometric mean of rabies VNA titres of dogs vaccinated with whole virus or ISCOM preparations and then challenged with street rabies virus. The VNA induced by the ISCOM preparations was significantly higher ($p < 0.012$) than that induced by the whole virus. The prechallenge VNA titres of dogs vaccinated with PM ISCOMs was significantly higher ($p < 0.021$) than those vaccinated with CVS ISCOM preparations. In general the postbooster response of dogs vaccinated with ISCOMs was higher than those vaccinated with whole virus, particularly for CVS groups ($p < 0.021$). The VNA response after challenge of dogs vaccinated with either ISCOM preparation was similar ($p > 0.750$). (——), PM ISCOM; (– – –), CVS ISCOM; (·····), CVS ISCOM (purified); (–·–·–), PM whole virus

Table 1 Mortality of mice vaccinated intraperitoneally with either one or two doses of G-ISCOM or two doses of HDCV (7 days apart) and challenged intracerebrally with challenge virus strain (CVS)

Group	Vaccine	No. of doses	Inoculated	Deaths (%)
1	ISCOM	1	40	5 (12.5)[a]
2	ISCOM	2	40	2 (5.0)[a]
3	HDCV	1	40	25 (62.5)[b]
4	HDCV	2	40	4 (10.0)[a]
5	Control	0	40	38 (95.0)[b]

[a] $p < 0.05$ compared with groups 3 and 5.
[b] $p < 0.001$ compared with groups 1, 2 and 4

Table 2 Mortality of mice inoculated in the footpad with a street rabies virus and then vaccinated intramuscularly with either ISCOM or HDCV

Group	Vaccine	No. of doses	Inoculated	Deaths (%)
1	ISCOM	1	30	16 (53.3)[b]
2	ISCOM	2	30	3 (10.0)[c]
3	ISCOM	3	48	3 (6.2)[c]
4	HDCV	3	30	21 (70.0)[b]
5	HDCV[a]	4	41	32 (78.0)[b]
6	Control	0	30	22 (73.3)[b]

[a] Eleven out of 52 mice died of rabies before the fourth vaccine dose; of the remaining 41 mice, 32 died of anaphylactic shock when the fourth dose was administered.
[b] $p < 0.001$ compared with groups 2 and 3.
[c] $p < 0.001$ compared with groups 1, 4, 5 and 6

RESULTS

Pre-exposure vaccination of mice

Since pre-exposure vaccination of dogs with either PM or CVS ISCOM preparations yielded a high level of immune response (*Figure 1*), the PM ISCOM was selected for comparison with the HDCV prepared from the same virus strain.

Ninety-five percent (38/40) of mice survived i.c. challenge after vaccination with two i.p. doses of ISCOM containing 360 ng rabies G-protein, and 90% (36/40) of mice vaccinated with two i.p. doses of 0.5 ml HDCV also survived. The difference in efficacy between two doses of ISCOM or HDCV was not significant ($p > 0.1$). Of the mice that received only one 360 ng i.p. ISCOM or 0.5 ml HDCV dose, 87.5% (35/40), and 37.5% (15/40), respectively, also survived and the difference between these vaccines is significant ($p < 0.05$) (*Table 1*).

Postexposure vaccination of mice

Of the 30 mice injected in the footpad with street rabies virus and vaccinated with three 0.1 ml doses of HDCV, most showed signs of rabies 2 days after the third vaccine dose. Twenty-one out of 30 vaccinated mice died of rabies, whereas only three of the 30 mice treated with three doses (120 ng) of ISCOM died. The difference in protection values between three doses of either vaccine is statistically significant ($p < 0.001$) (*Table 2*).

In a second group of 52 mice that received three 0.1 ml i.m. doses of HDCV, 11 (21%) died of rabies 4 days after the third dose was administered. When the fourth HDCV dose was administered on day 14, 32 of the remaining 41 mice (78%) died, with no signs or severe reaction, of generalized convulsion (anaphylactic shock) occurring immediately after their inoculation (*Table 2*).

Only three out of 48 challenged mice (6%) died after receiving four 120 ng i.m. doses of ISCOM, and 16 out

of 30 mice (53%) administered one dose of 120 ng ISCOM. Protection values of three doses of HDCV or one dose of ISCOM were not significantly different from the negative control ($p > 0.1$) (*Table 2*).

Pre-exposure vaccination of dogs

All dogs vaccinated with an ISCOM, regardless of the virus strain used for preparation of the ISCOM, and those vaccinated with whole virus were protected against a street rabies virus challenge, whereas all unvaccinated control dogs died of rabies. Pre- and postchallenge rabies VNA titres of dogs are depicted in *Figure 1*. Although the antigenic value of the ISCOM preparations and the whole virus inoculum were similar, the VNA induced by the ISCOM preparations was significantly higher ($p < 0.012$) (*Figure 1*). The geometric mean prechallenge VNA titre (GMT) of dogs immunized with PM whole virus vaccine (group 1) was 0.230 IU (range 0.2–0.4 IU), whereas the postchallenge titre was 1.222 IU (range 0.1–9.3 IU). The geometric mean prechallenge VNA titre of dogs vaccinated with the ISCOM prepared from PM virus (group 2) was 2.289 IU (range 0.9–5.7 IU), and the postchallenge titre was 9.359 IU (range 2.0–3.86 IU) (*Figure 1*). The prechallenge VNA of dogs in group 2 was significantly higher ($p < 0.021$) than in groups 3 and 4. In general the booster response of dogs vaccinated with ISCOMs was higher than those vaccinated with whole virus, particularly for groups 3 ($p < 0.021$) and 4 ($p < 0.021$). The booster response of dogs vaccinated with ISCOM preparations was similar ($p > 0.750$).

Five dogs vaccinated with whole virus (group 1) had a low detectable anti-N titre whereas the remaining 15 dogs given ISCOMs were negative (data not shown), indicating that the rabies G-protein preparation used in this experiment was relatively pure and did not induce any detectable amounts of antinucleocapsid antibody.

DISCUSSION

Limited information exists on experimental subunit rabies vaccines. However, recently rabies virus G-protein vaccinia–recombinant vaccines have been successfully used to immunize animals[50], and rabies G-protein presented in a virosome was also shown to be more immunogenic than that presented as protein micelle or killed vaccine[51].

In the present experiment, ISCOM vaccines prepared from two different rabies virus strains were compared with conventional human rabies vaccine in animal models (dogs and mice). No obvious differences in VNA response or protection were observed between the lectin-purified or the crude detergent-solubilized CVS ISCOM preparations, but the PM ISCOM had higher VNA titre than CVS, especially at 90 days after vaccination. One dose of 0.73 μg of either of the ISCOMs induced significantly higher VNA titre than a whole virus vaccine with similar antigen value. Most remarkable was the high anamnestic response in animals vaccinated with the ISCOMs and then challenged with a street rabies virus, signifying that a memory cell response was induced.

The efficacy of rabies vaccination has been shown in animals many times, and appears to correlate with the presence and degree of neutralizing-antibody titre[26,52]. In some experiments, however, it has been documented that animals that fail to elicit neutralizing-antibody titre after exposure to viral antigen may resist rabies virus challenge[53], whereas others which had neutralizing-antibody titre succumbed[26,54]. ISCOM-borne antigens induce cell-mediated immunity (CMI) efficiently[41] and they are unique (as shown with HIV-1 and influenza viral antigens) in their capacity to induce cytotoxic T-lymphocytes (CTL) which are $CD8^+$ and under the restriction of MHC class 1. This class of CTL has been shown to confer protection against many membrane viruses, indicating that CMI to rabies virus might also be important for protection[55,56].

Unlike other human viral vaccines, rabies vaccines are mainly used for postexposure treatment of exposed individuals, and the induction of virus-neutralizing antibody has been the parameter measured in judging the protection against rabies[52]. In humans and animals, however, administration of vaccine alone has rarely been demonstrated to prevent disease, unless anti-rabies serum (or globulin) was administered simultaneously[6,7,10,51,57–59]. Protective postexposure effect is not conferred by anti-rabies serum alone, indicating that vaccine activates protective mechanisms besides the neutralizing-antibody response[51,57–61]. Whether the protection provided by postexposure immunization is to a greater or lesser extent dependent on CMI is not known. However, it is shown that ISCOMs containing the F-protein of measles virus efficiently induce CMI in mice including the T_H1 type of cells which after adoptive transfer provided protection against i.c. virus challenge. These cells also produced IL-2 and gamma interferon[40]. It was also shown that envelope proteins of influenza virus and HIV-1 incorporated in ISCOMs induce IL-2 gamma interferon producing cells more efficiently in mice than micelles of the same antigen. In the case of influenza virus, ISCOMs were more efficient than the live virus (B. Morein, unpublished data).

In the present study, three 120 ng rabies G-protein ISCOM doses administered postexposure protected 90% of rabies-infected mice, whereas HDCV gave no protection, even though both antigens were prepared from the same vaccine strain. Two ISCOM doses were equally effective, indicating that rabies ISCOMs are effective in post-exposure treatment of mice without the additional application of anti-rabies globulin. The use of commercial vaccine, however, was not only ineffective in post-exposure treatment of rabies-infected mice but also caused fatal hypersensitivity reaction similar to the systemic allergic reactions, ranging from hives to anaphylactic shocks, reported in humans in both pre- and postexposure immunization[62–65]. Thus, with ISCOM vaccines it may be possible eventually to eliminate antiserum from postexposure rabies treatment, avoiding the expense and adverse reactions[61–65].

We have demonstrated that very low quantities of rabies G-proteins incorporated into ISCOM (without the additional application of anti-rabies globulin) protect mice against lethal rabies challenge when administered as a postexposure vaccine. Rabies ISCOM and whole virus vaccines were also successfully used for pre-exposure vaccination of dogs. Human postexposure immunization with ISCOMs may be possible, especially in areas where anti-rabies globulins may not be available for economic or technical reasons. Postexposure trials in other primates and other laboratory animals may be carried out to determine whether ISCOM subunit vaccines can be recommended for use in humans.

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