IMMUNOGENICITY OF IMMUNOSTIMULATING COMPLEXES OF JAPANESE ENCEPHALITIS VIRUS IN EXPERIMENTAL ANIMALS

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Summary. – Immunogenicity of immunostimulating complexes (ISCOMs) of Japanese encephalitis virus (JEV) were studied in mice, rabbits and monkeys. Two doses of JE ISCOMs elicited a strong immune response in mice with an uniform distribution in IgG subclasses. Different time intervals between the two doses of ISCOMs led to similar titers of antibodies. Rabbits and monkeys immunized with ISCOMs developed strong neutralizing immune, response. Mice immunized with ISCOMs demonstrated cell-mediated immunity (CMI) as evidenced by T cell proliferation and macrophage migration inhibition (MMI) assays.

Key words: immunostimulating complexes; Japanese encephalitis virus; immunogenicity; mice; monkeys; rabbits

Introduction

JEV causes an acute inflammatory disease of the central nervous system (CNS) and is of major concern in South-east Asia and the Indian subcontinent. A formalinized mouse brain vaccine produces a good seroconversion rate in humans after three doses, but it may cause allergic reactions in some cases (Anderson and Ronne, 1991).

ISCOMs have been described as structures containing amphipathic proteins assembled in a multimeric complex through hydrophobic interactions with adjuvant Quil A (Morein *et al.*, 1984). ISCOMs have been prepared from a large number of viral antigens and their strong immunogenicity has been documented (Classen and Osterhaus, 1992). Also ISCOMs prepared from cytomegalovirus, Epstein-Barr virus, influenza virus and human immunodeficiency virus (HIV) have been shown to stimulate CMI (Classen and Osterhaus, 1992). ISCOMs prepared from the envelope glycoprotein (Egp) of flavivirus have not been reported before. Hence ISCOM technology was applied to JEV to evaluate JEV ISCOMs as a candidate vaccine.

ISCOMs were prepared from JEV Egp (Yeolekar and Banerjee, 1996). This communication reports the humoral immune response of mice, rabbits and monkeys, and cellular immune response in mice immunized with JEV ISCOMs.

Abbreviations: BPL = beta propiolactone; CMI = cell-mediated immunity; CNS = central nervous system; Egp = envelope glycoprotein; ELISA = enzyme-linked immunosorbent assay; FCS = foetal calf serum; GM = geometric mean; HI = haemagglutination inhibition; HIV = human immunodeficiency virus; HRP = horseradish peroxidase; id = intradermal(ly); in = intranasal(ly); ip = intraperitoneal(ly); iv = intravenous(ly); ISCOM = immunostimulating complex; JEV = Japanese encephalitis virus; MMI = macrophage migration inhibition; MNL = mononuclear leukocytes; NI = neutralization index; PBS = phosphate buffered saline; RT = room temperature; sc = subcutaneous(ly); SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

Materials and Methods

Preparation of ISCOMs was done as described earlier (Yeolekar and Banerjee, 1996). Briefly, JEV Egp was obtained from purified JEV by lysis of the virions with detergent Triton X-100, followed by a step gradient centrifugation to separate Egp from the core. Dialysis of mixture of Egp-detergent in the presence of 0.2% Quil A (adjuvant) led to ISCOM formation. These ISCOMs

appeared as spherical cage-like particles (size 35 nm, 19.5 S). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) of ISCOMs indicated that only Egp was incorporated in ISCOMs. The amount of protein in ISCOMs was estimated in a standard way (Lowry *et al.*, 1951).

Immunization of mice. Two doses of 1.0 µg of ISCOMs were inoculated intraperitoneally (ip) in groups of 6 adult BALB/c mice at an interval of 2, 4, 6, 8 and 11 weeks. Each group was bled on day 7 after the second dose. Antibody titers of sera of individual mice were determined by enzyme-linked immunosorbent assay (ELISA) and geometric mean (GM) titers for each group were estimated. The significance of differences was analyzed by the t-test.

Immunization of rabbits. Two rabbits were immunized with 20 µg of ISCOMs, two rabbits with 1.0 ml of Kasauli JE vaccine (mouse brain-derived formalin inactivated vaccine), and one rabbit with 20 µg of BPL-inactivated purified JEV, by intradermal (id) route. The same dose was repeated 21 days after the first one. The rabbits were bled on days 15, 30, 54, 75 and 97 after the first dose. Normal rabbit serum was used as a control. The haemagglutination inhibition (HI) and ELISA antibody titers of sera obtained by immunization with JEV ISCOMs have been reported earlier (Yeolekar and Banerjee, 1996). The *in vitro* virus neutralization test was performed with these sera.

Immunization of monkeys. Two monkeys were immunized id with 30 µg of ISCOMs and one monkey with 30 µg of purified BPL-inactivated JEV. A second dose containing the same amount of antigen was administered after 30 days. Monkeys were bled on days 9, 15, 27, 40, 44, 51, 58 and 83 after the first dose. A serum obtained from a monkey before immunization was used as negative control. The obtained sera were tested in HI and *in vitro* neutralization test.

HI test was performed in microtiter plates according to the method described by Clarke and Casals (1958).

Neutralization test. Neutralization antibody titers of sera were estimated employing the method of varying dose of virus and constant dilution of serum (Shope and Sather, 1979). The neutralization indices (NI) of sera were obtained by subtracting virus titers in the presence of serum from virus titers in the presence of diluent.

ELISA. (a) Ig isotyping: Ten 4-week-old BALB/c mice were immunized ip with two doses of 2.5 µg of ISCOMs at an interval of 15 days and bled on day 7 after the second dose. The pooled serum was used in the test. Antisera for subisotyping were obtained from Hyclone Laboratories. IgG1, IgG2a, IgG2b, IgG3, IgM and IgA antibodies were detected by these antisera.

Microtiter plates (Immulon 2) were coated with JEV (0.5 µg/well) in carbonate buffer at 4°C overnight. Free binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline pH 7.2 (PBS) at 37°C for 30 mins. Immune mouse serum raised against ISCOMs and normal mouse serum were diluted 1:100 and added to 8 wells containing the antigen. The plates were incubated at 37°C for 1 hr. Fifty µl of typing antisera raised in rabbits (Hyclone Laboratories) were added to different wells and incubated at room temperature (RT) for 1 hr. Then 50 µl of anti-rabbit horseradish peroxidase- (HRP) conjugate and 200 µl of substrate solution containing ortho-phenylene diamine (2.5 mg/ml) and urea peroxide (0.2 mg/ml) was added to each well and incubated at RT for 15 mins. The reaction was stopped with 100 µl of 4N H_2SO_4 and A_{492} was read. The positive to negative (P/N) ratio for each IgG subclass was calculated. The total of all the P/N ratios for different classes and subclasses of Ig represented the total amount of JEV-specific Ig in the serum. Using the P/N ratios, the percentage of each Ig subclass was determined.

(b) Estimation of antibody titers of immune mouse sera was performed similarly to the isotyping procedure. Serum samples diluted two-fold in 1% BSA in PBS were added to virus-coated wells and probed with rabbit anti-mouse HRP-conjugate (Sigma). Normal mouse serum was used as negative control.

T cell proliferation assay in vitro. Six-week-old female BALB/c mice were inoculated ip with (a) 2.5 µg of ISCOms, (b) 2.5 µg of purified BPL-inactivated JEV, or (c) 10% JEV-infected mouse brain suspension. All mice were sacrificed on day 7 after immunization and their spleens were harvested. Cellular elements were teased out of spleens in RPMI medium, and mononuclear leukocytes (MNLs) were separated by Histopaque gradient centrifugation. MNLs were resuspended at a concentration of 2×10^6 cells/ml in RPMI medium containing 10% foetal calf serum (FCS). The purified BPL-inactivated JEV was diluted two-fold (in quadruplicates) in 96-well-plates to obtain a concentration of 5.0, 2.5, 1.26, 0.625, 0.3125 and 0.1525 µg/well of antigen. The MNL suspension (100 µl) was added to each well. ^{3}H-thymidine (1 µCi/well) was added 16 hrs before termination of the cultivation. The cells were harvested 4 days later onto glass fiber filter paper and the radioactivity was measured in a LKB Rackbeta scintillation counter using PPO- and POPOP-toluene scintillation cocktail. GM of the activity measured in four wells of each dilution was determined.

MMI assay was performed according to the method described by Kelkar and Banerjee (1978). Six-week-old BALB/c mice were immunized intravenously (iv) with either 2.5 µg of ISCOMs, 2.5 µg of purified inactivated JEV or saline. The spleens were harvested on the 7th day and MNLs were separated by Histopaque gradient centrifugation as given above. MNLs (2×10^6) were packed into thin capillaries, which were sealed from below using moulding clay and cut at the cell-medium interface. Cut portions of the capillaries were fixed in MMI assay chambers (shallow plastic wells with broad ridges) containing medium and the chambers were sealed with a coverslip. The chamber medium contained either (a) 100 HAU/ml JEV antigen (inactivated acetone-extracted JEV from infected mouse brains, (b) "normal" antigen (acetone extract of uninfected mouse brains), or (c) saline. The test was performed in quadruplicates. The cultures were incubated at 37°C for 18 hrs. The area of macrophage migration in each well was expressed in the weight of paper covering it and an average for each quadruplicate was determined. MMI (the ratio of macrophage migration in wells containing antigen to that in wells containing saline in %) was calculated.

Results

Distribution of different classes and subclasses of IgG in mice

The percentage of different Ig after immunization with ISCOMs was as follows: 28.11% of IgG1, 23.02% of IgG2a,

20.42% of IgG2b, 25.54% of IgG3, 1.8% of IgM and 1.08% of IgA. These results indicate an almost uniform distribution of IgG subclasses. The values for IgM and IgA were relatively very low.

Immune response in mice. Effect of dose interval

A plot of antibody titers (ELISA, GM) versus the dose interval is shown in Fig. 1. The highest titers were developed at an interval of 6 weeks between the two doses of ISCOMs. A short dose interval of 2 weeks and a long dose interval of 11 weeks elicited a weaker immune response in mice as compared to that elicited with a dose interval of 6 weeks. However, the difference between the dose interval of 2 weeks and 6 weeks was not statistically significant ($P = 0.062$).

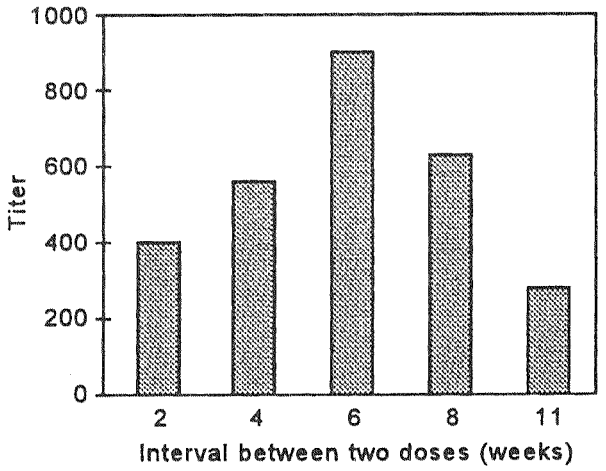

Fig. 1
Effect of various immunization dose intervals on antibody titers in mice

Immune reponse in rabbits

Neutralizing antibody titers, developed in rabbits immunized with different antigens, are summarized in Table 1. Two rabbits immunized with two 20 μg doses of ISCOMs at an interval of 21 days developed *in vitro* NI of 4.0 and 3.0, respectively, seven days after the second dose. The both rabbits immunized with 1.0 ml (human dose) of Kasauli JE vaccine (formalin-inactivated mouse brain-derived vaccine) developed NI of 5.0 as observed in the *in vitro* test. However, at the end of three months, the *in vitro* NI value of the both ISCOM-immunized rabbits was 4.0, while that of rabbits immunized with Kasauli JE vaccine was 3.0 (Table 1).

Table 1. Immune response in rabbits after immunization with ISCOMs

Antigen inoculated	NI				
	Days after the 1st dose				
	15	30	54	75	97
ISCOMs (20 μg)	0.0	4.0	3.0	3.0	3.0
ISCOMs (20 μg)	1.0	3.0	3.0	3.0	3.0
Kasauli vaccine (1.0 ml)	1.0	5.0	5.0	4.0	2.0
Kasauli vaccine (1.0 ml)	1.0	5.0	5.0	4.0	2.0
BPL-inactivated purified JEV (20 μg)	0.0	1.0	1.0	1.0	1.0

The second dose was inoculated on day 21 after the first dose.

Immune response in monkeys

Table 2 indicates HI titers and NI of sera obtained from monkeys immunized with ISCOMs and inactivated JEV. While one of the monkeys immunized with ISCOMs had high HI titers (1:640), the monkey immunized with inactivated JEV failed to elicit HI titers. NI values of 5.0 and 4.0, estimated by the *in vitro* neutralization test, indicated a strong neutralizing immune response in monkeys, immunized with 30 μg of ISCOMs, on the days 10 and 14 after the second dose. A monkey immunized with inactivated JEV elicited a weak neutralizing response (NI 3.0) on day 14 after the second dose as compared to that elicited in monkeys immunized with ISCOMs. These sera were also tested in the *in vivo* neutralization test. The *in vivo* NI values of 3.12 and 1.87 were obtained for the sera collected on day 14 after the second dose from the two monkeys immunized with ISCOMs. A serum obtained from a monkey immunized with purified inactivated JEV failed to neutralize the virus in the *in vivo* neutralization test (data not shown).

T cell proliferative response

Fig. 2 shows a plot of the amount of stimulating antigen versus the cpm of incorporated ^{3}H-thymidine for MNLs (GM of four values) obtained from mice immunized with ISCOMs, inactivated virus or live virus, respectively.

Maximum proliferative response was observed with 2.5 μg/ml antigen in *in vitro* stimulation. High cpm values of incorporated ^{3}H-thymidine in MNLs obtained from ISCOMs-immunized mice indicated active proliferation. Here, no secondary stimulation was observed in contrast to MNLs obtained from mice immunized with inactivated JEV or live virus.

Table 2. Immune response in monkeys after immunization with ISCOMs

Antigen inoculated	Days after the first dose															
	9		15		27		40		44		51		58		83	
	HI	NI	HI	NI	HI	NI	NI	HI	NI	HI	NI	HI	NI	HI	NI	HI
ISCOMs (30 μg)	<10	0.0	<10	0.0	10	2.0	80	5.0	320	5.0	160	4.0	80	3.0	ND	3.0
ISCOMs (30 μg)	<10	0.0	<10	0.0	<10	0.0	20	4.0	20	4.0	10	3.0	10	3.0	ND	3.0
Inactivated JEV (30 μg)	<10	0.0	<10	0.0	<10	0.0	<10	0.0	<10	3.0	<10	3.0	<10	2.0	ND	2.0

The second dose was inoculated on day 30 after the first dose.
HI = haemagglutination-inhibition titer. NI = neutralization index. ND = not done.

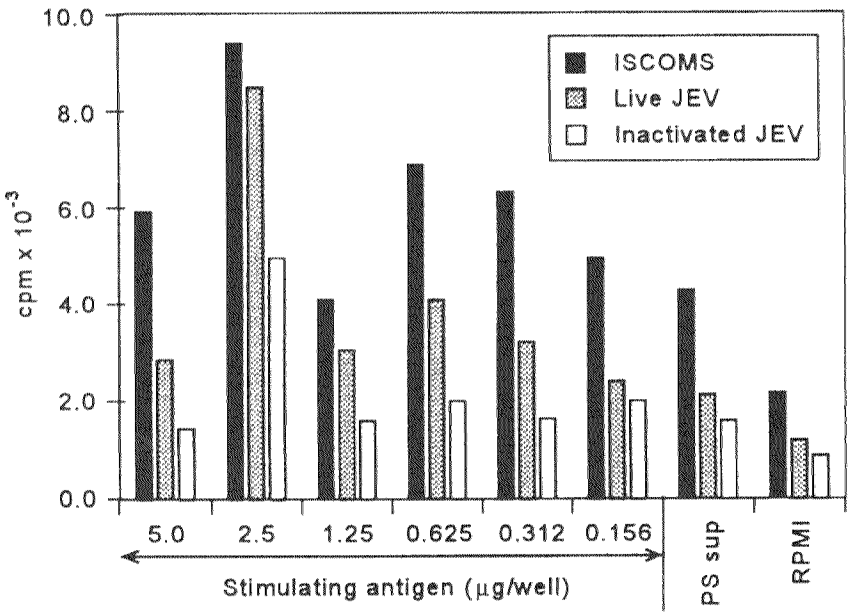

Fig. 2
T cell proliferative reponse in mice immunized with ISCOMs, live JEV or inactivated JEV
Stimulating antigen: inactivated JEV. PS sup = cell lysate of normal PS cells; RPMI = RPMI medium.

Macrophage migration inhibition (MMI)

MNLs from mice immunized with ISCOMs, inactivated purified JEV or saline were subjected to MMI assay. The results show (Table 3) that the MMI value for ISCOMs was 46.36% as compared to 16.95% for inactivated JEV. Value of 7 – 8% were observed in the presence of "normal" antigen in mice immunized with ISCOMs, inactivated virus or saline.

Discussion

The manner in which virus antigens are presented to the immune system of animals can influence the Ig classes and subclasses of the antibody response (Balkovic *et al.*, 1987).

Lovgren (1988) showed that ISCOMs containing haemagglutinin and neuraminidase glycoproteins of influenza virus PR8 strain induced a classic serum antibody response in mice after both sc and in immunization, with an early IgM response, followed by an IgG response. The antibodies were also shown to be evenly distributed in all IgG isotypes i.e. IgG1, IgG2a, IgG2b and IgG3.

In the present study of JEV ISCOMs, an even distribution of antigen-specific antibodies was observed within the IgG subclasses. Lower amount of IgM could indicate a secondary immune response. The even distribution of the serum IgG isotypes induced by ISCOMs is of particular interest for a prospective vaccine. It demonstrates that the adjuvant Quil A administered in a low dose together with the antigen in the form of ISCOMs induces a complete spectrum of IgG isotypes.

Table 3. MMI for various immunizing antigens

Immunizing antigen	Antigen in the chamber medium	Average weight of paper	Inhibition (%)
ISCOMs	JEV	14.75	46.36
	"Normal" antigen	25.50	7.27
	Saline	27.50	–
Inactiveted	JEV	23.66	16.95
JEV	"Normal" antigen	26.00	8.77
	Saline	28.50	–
Saline	JEV	30.25	11.88
	"Normal" antigen	31.66	7.77
	Saline	34.33	–

Hoglund *et al.* (1989) reported that a longer interval between the doses of ISCOMs was necessary to develop significant immune reponse. They observed that with ISCOMs, containing HIV gp160, an interval of 6 weeks was optimal, while no or dubious booster effect was observed when the interval was only 2 weeks.

In experiments on the effect of the interval between the two immunization doses of JEV ISCOMs on the immune response in mice, the difference observed in antibody titers (ELISA) for dose intervals of 2 and 6 weeks was not statistically significant. This indicated that a second dose given 2 or 6 weeks later has approximately the same booster effect on the immune response.

Two doses of 20 μg of JEV ISCOMs elicited in rabbits a strong immune response as indicated by the *in vitro* neutralization titers. Similarly, monkeys inoculated with ISCOMs developed a strong immune response as detected by the HI and neutralization tests.

The role of CMI in flavivirus infection is not clearly understood. There are reports on the ability of some flaviviruses to induce a delayed type hypersensitivity reaction (Pang *et al.*, 1982), MMI (Kelkar and Banerjee, 1978) and cytotoxic T lymphocyte response (Murali Krishna *et al.*, 1995). Our studies suggest that a significant amount of T cell proliferative response could be stimulated by immunizing mice with JEV ISCOMs. This stimulation was higher than that observed in MNLs obtained from mice immunized with live or inactivated JEV. Saponins have been shown to stimulate markedly the clonal expansion of immunocompetent cells in the mesentric lymph nodes and spleen (Campbell and Perbaye, 1992). The higher values of incorporation of ^{3}H-thymidine in cells grown in the absence of secondary stimulation could be attributed to the basal activity of saponins present in ISCOMs.

MMI is an indicator of the delayed type hypersensitivity reaction (Rocklin *et al.*, 1970). MMI is an index of CMI reaction and evaluates the potency of the vaccine by showing the presence of sensitized T cells in the immunized host. The MMI test has been used to study the cross-reactivity among flaviviruses in CMI (Kelkar and Banerjee, 1978).

In our experiments, the marked MMI with JEV ISCOMs was observed in the presence of homologous virus. This indicated that along with a good humoral immune response a strong CMI response was induced by JEV ISCOMs.

The evaluation of JEV ISCOMs indicates that (1) the incorporation of JEV Egp into ISCOMs enhanced its immunogenicity, (2) two doses of JEV ISCOMs stimulated a strong neutralizing immune response, and (3) JEV ISCOMs were capable of eliciting CMI.

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