

Enhanced protection of mice against Japanese encephalitis virus infection by combinations of monoclonal antibodies to glycoprotein E

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Summary. – In the present study, the protective effect of various combinations of four monoclonal antibodies (MAbs) to glycoprotein E (gpE) of Japanese encephalitis virus (JEV) on the JEV-infected mice was studied. The MAbs were characterized as hemagglutination-inhibition-positive and JEV-specific (Hs). In the protective experiment, mice were first administered single MAbs or their combinations intraperitoneally (i.p.) and 24 hrs later infected with the virus intracerebrally (i.c.). The results showed that single MAbs protected the mice to the extent of 45–65%, while combinations of two or three MAbs gave 85–90% or 100% protection, respectively. The enhanced effect of combinations of several Hs MAbs might be due to the sharing of neutralization epitopes recognized by the Hs MAbs. These results suggested that a combination of at least three epitopes represented by the Hs MAbs should be included in an effective JEV vaccine.

Keywords: Japanese encephalitis virus; monoclonal antibodies; glycoprotein E; mice; protection

Introduction

JEV is one of the mosquito-borne flaviviruses (the genus *Flavivirus*, the family *Flaviviridae*) that causes frequent epidemics of acute encephalitis throughout South-east and Western Pacific regions (Vaughn and Hoke, 1992; Monath and Heinz, 1996; Solomon *et al.*, 2003). The mortality as high as 40% was recorded in some of the Japanese encephalitis (JE) affected areas. Moreover, many survivors face some neurological problems and complications (Rodrigues, 1984; Solomon *et al.*, 2003). Since 1995, the disease has also

emerged in non-Asian region such as Northern Australia (Hanna *et al.*, 1996, 1999). The situation in South-east Asia, however, is further complicated by the overlapping epidemics of JEV and dengue virus (DENV) as well as sporadic cases of West Nile virus (WNV) infections detected in some of the affected areas particularly in India (George *et al.*, 1984). These three antigenically-related viruses pose serious hazard to the public health and complicate to a great extent both vaccination and host immunologic response in endemic areas as well (Carey and Meyers, 1968).

The gpE of flaviviruses contains most of the antigenic epitopes that induce various biological functions including hemagglutination and neutralization and also shows antigenic activity that ranges from specific to the cross-reactive (Peiris *et al.*, 1982; Kimura-Kuroda and Yasui, 1983; Gould *et al.*, 1986). Mapping of gpE for the antigenic epitopes on JEV strain 733913 employing MAbs raised earlier in our laboratory have shown existence of five domains represented by the MAb groups belonging to HAI-positive, virus specific (Hs); HAI-negative, virus-specific (NHs); HAI-positive flavivirus cross-reactive (Hx); HAI-negative flavivirus cross-reactive (NHx) and HAI-negative auto-reactive (NHA) MAbs (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988).

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Abbreviations: DENV = dengue virus; gp E = glycoprotein E; HAI = hemagglutination-inhibition; Hs = HAI-positive, virus specific; Hx = HAI-positive, flavivirus cross-reactive; i.c. = intracerebrally; i.p. = intraperitoneally; JE = Japanese encephalitis; JEV = Japanese encephalitis virus; MAb(s) = monoclonal antibody(ies); NHA = HAI-negative, auto-reactive; NHs = HAI-negative, virus specific; NHx = HAI-negative flavivirus cross-reactive; WNV = West Nile virus

The four MABs representing Hs epitopes are neutralizing and mainly protective in mice (37–70% protection), when administered alone as ascitic fluid 1 day before JEV infection (Gupta *et al.*, 2003). However, their additive or enhanced effect has not been examined yet. In the present study, we attempted to determine the protective effect of combinations of two or three MABs on the JEV-infected mice.

Materials and Methods

Virus and mice. JEV strain 733913 and Swiss mice employed in this study were described elsewhere (Ghosh *et al.*, 1989; Gupta *et al.*, 2003). In brief, the virus was maintained by i.c. passage in 2-day-old suckling mice and the stock virus was stored at -70°C. Randomly bred 4-week-old mice were employed in this study as approved by the Institutional Animal Ethics Committee at the National Institute of Virology, Pune, India.

Monoclonal antibodies. MABs against gpE of JEV strain 733913 prepared in our laboratory and characterized initially for their reactivity with JEV, DENV-2 and WNV were employed also for epitope mapping of gpE of JEV (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988). These MABs were grouped as Hs (4 MABs), NHs (2 MABs), Hx (5 MABs), NHx (3 MABs) and NHA (2 MABs). Ascitic fluids were obtained from pristane-primed BALB/c mice after inoculation of the hybrid cells. An antiserum against JEV was raised in immunized mice by inoculation of Ehrlich's ascitic tumors as per standard procedure.

Purified MAB IgGs. Purified IgGs were obtained from the ascitic fluids of MABs by ammonium sulphate precipitation followed by the Sepharose-protein A chromatography. MAB Hs-1 was of subtype IgG1, while remaining three MABs Hs-2, 3, and 4 belonged to the

subtype IgG2a. IgGs were also obtained similarly from the normal mouse serum and JEV mouse antiserum. The antibody protein concentration was determined and adjusted to 1 mg/ml with PBS pH 7.2. The HAI, neutralization test, ELISA, and competitive ELISA were performed as described in detail previously (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988; Gupta *et al.*, 2003, 2008). The antibody titres (expressed as 1/log10) of the four Hs MABs used in the present study ranged in different assays from 3.8 to 4.7 in HAI test, 3.7 to 5.0 in ELISA, in virus - neutralization *in vitro* from 3.0 to 3.7, whereas *in vivo* neutralization showed 3+ to 4+ protection in mice (3+ = 60% to 80%, 4+ = 80% to 100% protection). The 4 Hs MABs showed reciprocal blocking in competitive ELISA, but different epitopes were assigned to them due to the differences in antibody-binding enhancement (Cecilia *et al.*, 1988).

Protection immunization. About 4-week-old Swiss mice were divided into groups with 20 animals and administered combinations of three MABs i. p. (0.3 ml) with 100 µg of purified IgGs of each MAB per mouse. For two MABs combinations, 100 µg of normal IgGs was injected along with the MABs. The mice were challenged i.c. with 100 LD₅₀ of the virus 24 hrs later. Groups of mice administered with Hs MAB singly or with JEV antiserum (100 µg IgGs per mouse) along with the injection of 200 µg of normal IgGs to obtain the same level of administered IgG in each animal. Also, an additional group of mice administered normal IgG (200–300 µg/mouse) or normal mouse serum (diluted 1:10) was included with each experimental group as a negative control. The mice were observed for 21 days and the mortality expressed as number of mice died/ total number of the mice tested (in %) was compared with that of control mice.

Results and Discussion

The mortality of mice treated with single MAB or MABs in various combinations is presented in Table 1. Any of the 4 MABs IgGs protected the mice since the mortality of 35–55% was recorded in comparison to the controls (20/20; 100%). In contrast, combinations of MABs showed better protection and the mortality 10–15% or no mortality at all was recorded with any of the two or three MABs, respectively. Initially, the mice administered 100 µg of purified JEV antiserum IgG were protected to 100% and therefore, the same dose of the purified MABs IgGs was used in the protection experiment. However, different effect of the single MAB IgGs or combinations of two MABs in higher doses remains to be elucidated.

The mortality of mice treated with single Hs MAB IgG or with two MABs was further confirmed by the isolation of virus from their brains. However, a few of the survived mice treated with single or two MABs showed early signs of sickness such as a slight dullness, consuming less food, and slightly rough fur. These symptoms disappeared gradually and no virus was isolated from

Table 1. Mortality of JEV-infected mice protected with single MABs to JEV gpE and their combinations

Mice administered		Mortality (%)
No MAB (control)	Normal IgG	20/20 (100)
Single MAB ^a	Hs-1	8/20 (40)
	Hs-2	11/20 (55)
	Hs-3	10/20 (50)
	Hs-4	7/20 (35)
Combinations of two MABs ^b	Hs-1 + Hs-2	3/20 (15)
	Hs-1 + Hs-4	
	Hs-2 + Hs-3	
	Hs-2 + Hs-4	
	Hs-1 + Hs-3	
Combinations of three MABs	Hs-1 + Hs-2 + Hs-3	2/20 (10)
	Hs-1 + Hs-3 + Hs-4	
	Hs-2 + Hs-3 + Hs-4	

Individual MABs were administered in 100 µg per mouse. ^aIn addition, 200 µg of normal IgG per mouse was given. ^bIn addition, 100 µg of normal IgG per mouse was given.

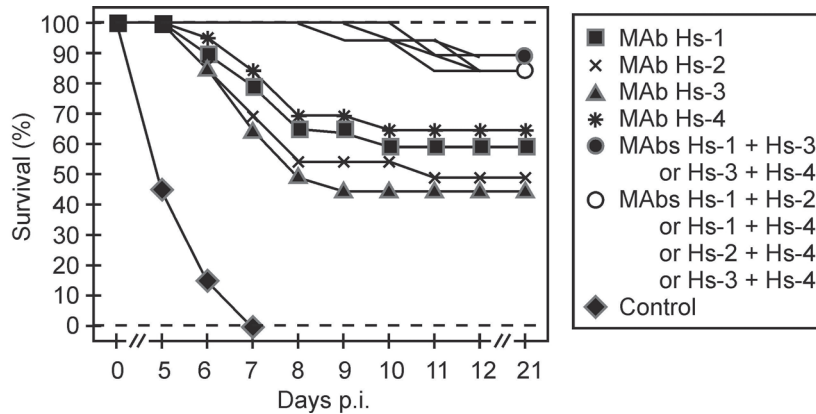


Fig. 1

Protective effect of various combinations of MAbs to JEV gpE on the JEV-infected mice

Control mice were given normal IgG or normal mouse serum.

the brains of recovered mice collected 21 days after the virus inoculation.

Fig. 1 depicts the survivors (in %) on different days p.i. with JEV among the mice administered with single Hs MAbs or with their combinations.

The characterization of MAbs directed against gpE of JEV has earlier indicated a critical neutralization site that was recognized by all the 4 Hs MAbs used in the present study (Kedarnath *et al.*, 1986). This site though seemed to be in close proximity (juxtaposition) to the epitopes represented by the NHs and NHx group of MAbs, but spatially far away to both Hx and NHA group of MAbs (Cecilia *et al.*, 1988). This was further confirmed by *in vivo* experiments, since any of the four Hs MAbs administered protected mice to an extent from 37% to 70% against lethal JEV infection, whereas the protection was minimal, if any, with the remaining MAbs including NHs and NHx group of MAbs (Gupta *et al.*, 2003).

In the present study, the treatment with a single MAb resulted in 45–65% protection, whereas the combinations of two or three MAbs produced 85–90% or 100% protection, respectively. Thus, the combinations of any three Hs MAbs produced enhanced protective effect similar to the JEV antiserum that might be due to the sharing of neutralization epitopes recognized by the 4 Hs MAbs. Clearly, to achieve a desirable effect in protection of mice against the JEV infection, the combinations of at least three MAbs have to be administered. In view of the fact that no specific therapies are available for the treatment of JE, the present findings gain importance in further possibilities of employing antibodies in the treatment of JE cases. Also a combination of at least three epitopes represented by the Hs MAbs should be included in an effective JEV vaccine. This will obviate a presence of any cross-reactive antibod-

ies that might represent the risk factor, particularly in the areas with flavivirus epidemics (Kimura- Kuroda and Yasui, 1988; Gupta *et al.*, 2003, 2009).

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