Protection of mice against Japanese encephalitis virus group II strain infections by combinations of monoclonal antibodies to different antigenic domains on glycoprotein E

Ashok Kumar Gupta,
Attiyaril Abraham Koshy ,
Vaibhavi Jawahar Lad

National Institute of Virology, Pune, India

Abstract

A combination of at least three hemagglutination-inhibition-positive (HAI) and virus-specific (Hs) monoclonal antibodies (MAbs) to glycoprotein E (gpE) of Japanese encephalitis virus (JEV) fully protected (100%) mice against JEV strain 733913 infections (group I). However, these representative epitopes are reported to have been lost on JEV group II strains. In the present study, therefore, the protective effect of various combinations of anti-gpE MAbs representing antigenic epitopes other than Hs was studied on mice infections with JEV group II strains: JEV strains 641686 and 691004. MAbs used in the protective experiments were characterized as HAI-negative virus-specific (NHs) and HAI-positive flavivirus cross-reactive (Hx). Additionally, one of the Hs MAbs (MAb Hs-3) was included in the experiments. Mice were first administered single MAbs or their combinations intraperitoneally and 24 h later, infected with the virus intracerebrally. Protection rates of 70-75% were obtained with a combination of four MAbs: MAbs NHs-1, Hx-1, Hx-3 and Hs-3. However, protection rates of only 20-40% were obtained with three MAbs but none was observed with single or two MAbs. There was, however, a substantial increase in mice survival. The protective effect of several combinations of anti-gpE MAbs representing different antigenic epitopes might be due to the enhancement of binding within the same group and also between different MAb groups. The present results indicate that NHs and Hx epitopes should be incorporated with three Hs epitopes in a JEV vaccine that would have an added advantage, particularly in the flaviviral endemic areas with JEV strain variations.

Introduction

Japanese encephalitis virus (JEV, the genus *Flavivirus* and family *Flaviviridae*), belongs to

a mosquito-borne flavivirus group consisting of some 66 antigenically-related viruses.¹ The virus has gained considerable importance as a human pathogen with an increase in frequency of epidemics of viral encephalitis as recorded throughout South-East Asia and Western Pacific regions.²⁻⁴ Mortality as high as 40% was recorded in some of the Japanese encephalitis (JE) affected areas. Furthermore, many survivors face some neurological problems and complications.^{4,5} Since 1995, the disease has also been shown to emerge in non-Asian regions such as Northern Australia.^{6,7} The situation in South-East Asia, however, is further complicated by overlapping epidemics of JEV and dengue virus (DENV) as well as sporadic cases caused by West Nile virus (WNV). These all pose a serious hazard to public health.^{5,8} This has greatly complicated both vaccination and host immunological responses.⁹ The glycoprotein E (gpE) of flaviviruses contains most of the antigenic epitopes which induce various biological functions including hemagglutination and neutralization, and show antigenic reactivity that ranges from specific to cross-reactive.¹⁰⁻¹² Mapping of gpE for antigenic epitopes on JEV strain 733913 (group I of Indian origin) employing monoclonal antibodies (MAbs) raised earlier in our laboratory have shown the existence of five domains represented by the MAb groups belonging to hemagglutination-inhibition-positive (HAI)-positive (Hs), HAI-negative virus-specific (NHs), HAI-positive (Hx), HAI-negative flavivirus cross-reactive (NHx) and HAI-negative auto-reactive (NHA) MAbs.¹³⁻¹⁴ Furthermore, 21 JEV strains analyzed for the antigenic epitopes employing these MAbs revealed 15 strains with complete Hs epitope functional activity and neutralized by the Hs MAbs. These were placed in group 1.¹⁵ The remaining 6 JEV strains that have lost their neutralization ability with most of the Hs MAbs and neutralized by the MAbs representing NHs and Hx epitopes were grouped as group II strains. Also in protection experiments in mice with four Hs MAbs administered alone, 45-65% protection against JEV strain 733913 was noticed which was enhanced to 85-90% and 100% protection when the MAbs were given in combinations of two or three Hs, respectively.^{16,17} In contrast, no protection with Hs and another group of MAbs against the JEV group II strain infections was noticed except an increase in mice survival with the NHs and Hx MAbs, and to some extent also with MAb Hs-3.^{18,19} However, their additive or enhanced effect has not yet been examined.

The present study was, therefore, undertaken to determine the protective effect of combinations of two or more MAbs on mice infections with JEV group II strains (strains 641686 and 691004).

Correspondence: Ashok K. Gupta, Centre for Biotechnology, Pravara Institute of Medical Sciences, Loni 413736, India.
Tel. +91.9960.0712.91 - Fax: +91.2422.2734.13.
E-mail: drashok.gpt@gmail.com

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Materials and Methods

Mice and viruses

The details of the Swiss mice are given elsewhere.^{16,20,21} Randomly bred 4-week old mice were used in this study as approved by the Institutional Animal Ethics Committee at the National Institute of Virology, Pune, India. Two JEV strains (641686 of Indian origin and 691004 of Sri Lankan origin) were characterized as group II JEV strains on the basis of loss of Hs epitope functional activity.^{15,18,19} The JEV strain 733913 of Indian origin that belonged to group I strains of JEV was included in all the experiments. In brief, the virus was maintained by intracerebrally (i.c.) passage in 2-day old suckling mice and the stock virus was stored at -70°C.

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 used also for epitope mapping of gpE of JEV.^{13,14} These MAbs were grouped as Hs (4 MAbs), NHs (2 MAbs), Hx (5 MAbs), NHx (3 MAbs) and NHA (2 MAbs). Two Hx (Hx-1 and Hx-3) MAbs used in the experiments were of subtype IgG2b while MAbs NHs-1 and Hs-3 belonged to subtypes IgG1 and IgG2a, respectively. Ascitic fluids were obtained from pristane-primed BALB/c mice after inoculation of the hybrid cells. An antiserum against JEV was also raised in immunized mice by inoculation of Ehrlich's ascitic tumors as per standard procedure.

Purified monoclonal antibodies immunoglobulin G

Purified IgGs from the ascitic fluids of MABs, as well as from the normal mouse serum and JEV antiserum, were obtained by ammonium sulphate precipitation followed by Sepharose-protein A chromatography.¹⁷ Antibody protein concentration was determined and adjusted to 1 mg/mL with PBS pH 7.2. The HAI, neutralization test and enzyme-linked immunosorbent assay (ELISA) were performed as described in detail previously.^{13,14,16,20} The antibody titres (expressed as 1/log10) of the four MABs used in the present study were ranged in different assays from 3.8 to 4.7 in the HAI test (except for NHs-1, <1.0), 3.7 to 8.0 in ELISA and from 2.8 to 3.2 (except for Hx-3, <1.0) in *in vitro* virus-neutralization. MAB Hs-3 has protective abilities in mice against JEV strain 733913 in a high dose (100 LD₅₀), whereas MAB NHs-1 showed protection only against a low dose (5 LD₅₀). In contrast, MAB Hx-1 though neutralizing was non-protective and MAB Hx-3 achieved no protection with both JEV doses.

Protection experiments

The experiments were carried out as described elsewhere.^{16,17,20} In brief, 4-week old Swiss mice were divided into groups of 20 animals and administered a combinations of 4 MABs intraperitoneally (total 0.4 mL) with 100 µg of purified IgGs of each MAB per mouse. For 2 or 3 MAB combinations, 200 µg or 100 µg of normal IgGs were injected along with the MABs, respectively. Twenty-four hours later, the mice were challenged i.c. with 100 LD₅₀ of the virus. Groups of mice were administered singly with MABs NHs-1, Hx-1, Hx-3 or MAB Hs-3, or with JEV antiserum (100 µg IgGs per mouse) along with the injection of 300 µg of normal IgGs to obtain the same level of administered IgG in each animal. Also, an additional group of mice were administered normal IgG (300-400 µg/mouse) or normal mouse serum (diluted 1:10) in 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 (%) was compared with that of control mice. Average survival time (AST) of the dead mice in days was further determined as described previously.^{16, 20} However, those surviving the virus challenge till days 21 were excluded from the analysis.

Results

Challenge with Japanese encephalitis virus strain 733913

The effect of MABs administered singly on the homologous JEV strain 733913 has already

been examined in mice and the results were similar to those obtained in the present study.^{16,20} Those mice challenged with homologous virus served as positive control to the mice challenged with heterologous viruses. The results are invariably similar and, therefore, are not presented.

Challenge with Japanese encephalitis virus, strains 641686

and 691004

The mortality of mice treated with single MAB or MABs in various combinations is presented in Tables 1 and 2. Any of the 4 MABs IgGs or even combinations of 2 MABs did not protect the mice since 100% (20 of 20) mortality like in the controls (20 of 20; 100%) was recorded. In contrast, 60-80% and 25-30% mortality was recorded with combinations of 3 or 4 MABs, respectively. Initially, the mice administered

Table 1. Mortality of Japanese encephalitis virus (strain 641686)-infected mice passively given single monoclonal antibodies to Japanese encephalitis virus glycoprotein E and their combinations

Mice administered		Mortality (%)
No MAB (control)	Normal IgG	20/20 (100)
Single MAB*	NHs-1	20/20 (100)
	Hs-3	20/20 (100)
	Hx-1	20/20 (100)
	Hx-3	20/20 (100)
Combinations of two MABs°	Hs-3+Hx-1	20/20 (100)
	Hs-3+Hx-3	20/20 (100)
	Hs-3+NHs-1	20/20 (100)
	Hx-1+Hx-3	20/20 (100)
	Hx-1+NHs-1	20/20 (100)
	Hx-3+NHs-1	20/20 (100)
Combinations of three MABs#	Hs-3+Hx-1+Hx-3	12/20 (60)
	Hs-3+Hx-1+NHs-1	16/20 (80)
	Hx-1+Hx-3+NHs-1	12/20 (60)
	Hs-3+Hx-3+NHs-1	15/20 (75)
Combinations of four MABs	Hs-3+Hx-1+Hx-3 +NHs-1	6/20 (30)

MABs, monoclonal antibodies; IgG, immunoglobulin G; Hs, HAI positive virus-specific; NHs, HAI negative virus-specific; Hx, HAI-positive flavivirus cross-reactive. Individual MABs were administered in 100 µg per mouse. In addition, 300 µg of normal IgG per mouse; 200 µg of normal IgG per mouse and 100 µg of normal IgG per mouse were given into mouse groups *, ° and #, respectively.

Table 2. Mortality of Japanese encephalitis virus (strain 691004)-infected mice passively given single monoclonal antibodies to Japanese encephalitis virus glycoprotein E and their combinations.

Mice administered		Mortality (%)
No MAB (control)	Normal IgG	20/20 (100)
Single MAB*	NHs-1	20/20 (100)
	Hs-3	20/20 (100)
	Hx-1	20/20 (100)
	Hx-3	20/20 (100)
Combinations of two MABs°	Hs-3+Hx-1	20/20 (100)
	Hs-3+ Hx-3	20/20 (100)
	Hs-3+NHs-1	20/20 (100)
	Hx-1+Hx-3	20/20 (100)
	Hx-1+ NHs-1	20/20 (100)
	Hx-3+ NHs-1	20/20 (100)
Combinations of three MABs#	Hs-3+Hx-1+Hx-3	13/20 (65)
	Hs-3+Hx-1+NHs-1	14/20 (70)
	Hx-1+Hx-3+NHs-1	13/20 (65)
	Hs-3+Hx-3+NHs-1	16/20 (80)
Combinations of four MABs	Hs-3+Hx-1+Hx-3 +NHs-1	5/20 (25)

MABs, monoclonal antibodies; IgG, immunoglobulin G; Hs, HAI positive virus-specific; NHs, HAI negative virus-specific; Hx, HAI-positive flavivirus cross-reactive. Individual MABs were administered in 100 µg per mouse. In addition, 300 µg of normal IgG per mouse; 200 µg of normal IgG per mouse and 100 µg of normal IgG per mouse was given into mouse groups *, ° and #, respectively.

100 µg of purified JEV antiserum IgG were protected 100% against JEV group I and group II strains; therefore, the same dose of the purified MAb IgGs was used in the protection experiment. Although no statistical comparison with the controls (AST 5.4-5.5 days) was made, an increase in survival of the mice was noticed ranging from half a day to almost three days with the single MAbs. The pronounced effect was observed by the MAbs NHs-1 (AST 8.3 days) and Hx-3 (AST 7.7 days) against JEV strain 641686, while the increase was only marginal with MAbs Hx-1 (AST 6.2 days) and Hx-3 (AST 6.1 days) against JEV strain 691004. Similarly, with 2 or 3 MAb combinations, an increase in survival of the mice was noticed with either of the 2 JEV strains ranging from approximately 4-7 days (AST 8.3-12.3 days) and approximately seven to almost ten days (AST 12.8-15.3 days), respectively. In contrast, with 4 MAb combinations, the survival of mice was enhanced by approximately 11 to over 12 days with both of the JEV strains: 641686 (AST 16.3 days) and 691004 (AST 17.9 days).

The mortality of mice treated with single MAb IgG or combinations of 2 or more MAbs was confirmed by the isolation of virus from their brains. However, a few of the survived mice treated with 3 or 4 MAbs showed early signs of sickness, such as a slight dullness, consuming less food, and having slightly rough fur. These symptoms gradually disappeared and no virus was isolated from the brains of recovered mice collected 21 days after the virus inoculation. Figures 1 and 2 show the survivors (%) on different days p.i. with JEV strains 641686 and 691004 among the mice administered with single MAbs or with their combinations.

Discussion and Conclusions

The gpE is a major flaviviral antigen which binds to cellular receptors and mediates cell membrane fusion. It contains an array of epitopes that elicit virus neutralizing and non-neutralizing antibodies.^{10-12,22,23} The protective efficacy of an anti-gpE specific MAb is, therefore, directly related to its ability to neutralize the virus infectivity.^{16,20,24} 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 on gpE of JEV strain 733913 and these MAbs protected the mice against lethal virus challenge.^{13,17,20} However, JEV group II strains were neither neutralized nor the mice infected with JEV group II strains protected by the Hs MAbs except for some increase in mice survival with MAb Hs-3.^{19,20} As these JEV group II strains could still be neutralized with some of the MAbs belonging to the NHs and Hx groups, although no protection was noticed with the

MAb given alone except for an increase in mice survival.^{19,20} Therefore, their MAbs combinations were examined for additive or enhanced protective effect.

In the present study, 70-75% protection was achieved with a combination of 4 MAbs *i.e.*: MAbs NHs-1, Hx-1, Hx-3 and Hs-3. Protection was only 20-40% with the 3 MAbs and none with single or 2 MAbs except for an increase in mice survival against 2 JEV group II strains 641686 and 691004 infections. Although no statistical comparisons of AST values obtained in control and experimental group,

the increase in animal survival seemed to be directly related to the combinations of MAbs used. Addition of another MAb or 2 may produce a desirable effect (up to 100% protection) but this, however, remains to be clarified. Furthermore, JEV group II strains seemed to have emerged in different ecological situations over a number of years in the flavivirus endemic areas.¹⁵ A laboratory set-up for the selection of a neutralization-escape variant of the JEV strain 733913 against MAb Hs-1 showed that antibody pressure seemed to be one of the important factors in the evolution of

Figure 1. Protective effect of various combinations of monoclonal antibodies to Japanese encephalitis virus glycoprotein E on Japanese encephalitis virus (strain 641686) infected mice. Control mice were given normal immunoglobulin G or normal mouse serum.

Figure 2. Protective effect of various combinations of monoclonal antibodies to Japanese encephalitis virus glycoprotein E on Japanese encephalitis virus (strain 691004) infected mice. Control mice were given normal immunoglobulin G or normal mouse serum.

JEV group II strains.²⁵ Therefore, the presence of epitopes unique to these strains cannot be ruled out and it will, therefore, be worthwhile examining this possibility by carrying out the protective experiments using MABs prepared against some of the JEV group II strains. Interestingly, MAB Hx-3, which is non-neutralizing, also showed an increase in mice survival and/or protection when administered in combination with neutralizing MABs. Studies by Gould *et al.*¹² using neutralizing and non-neutralizing MABs against yellow fever virus (YFV) suggested that the ability of an antibody to protect the mice passively against the i.c. virus challenge depends on the virus neuro-virulence. Also, protection afforded by a non-neutralizing MAB against YFV was the indication of the involvement of other factors rather than neutralization alone which might be responsible for such an effect of MAB Hx-3 against JEV group II strains in mice.¹² It was shown that MABs can induce conformation-dependent changes in the viral epitopes.²⁶⁻²⁸ In the present study, therefore, the role of such changes in protection cannot be ruled out. In view of the fact that no specific therapies are available for the treatment of JE, the present findings gain importance in offering further possibilities of using antibodies in the treatment of JE cases.¹⁷ The results also indicate that NHs and Hx epitopes should be added to 3 Hs epitopes in a JEV vaccine that would, therefore, have an added advantage, particularly in endemic areas with JEV strain variations.

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