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Early death of Japanese encephalitis virus-infected mice administered a neutralizing cross-reactive monoclonal antibody against glycoprotein E

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Summary. – In the present study, the effect of two haemagglutination-inhibition (HAI)-negative auto-reactive (NHA-1 and NHA-2) monoclonal antibodies (MAbs) against glycoprotein E (gpE) of Japanese encephalitis virus (JEV) administered 1 day before or 2 days after intracerebral (i.c.) inoculation of JEV was studied in mice. Of the two MAbs that cross-reacted with West Nile virus (WNV) and histones, the first one (NHA-1) neutralized JEV, while the second one was non-neutralizing. NHA-1 MAb given intraperitoneally (i.p.) 1 day before virus infection induced early death by about 2 days in comparison to controls, whereas mice administered HAI-positive anti-gpE JEV specific MAbs (Hs-1 or Hs-4) were invariably protected. In contrast, MAb NHA-2 failed to produce any effect in mice. Since the similar virus titers were recorded in the brains of experimental and control infected mice, the present results indicated a modification of the biological activity of JEV by the pre-existing MAb NHA-1 that might be leading to an early death of mice.

Keywords: Japanese encephalitis virus; neutralizing cross-reactive monoclonal antibody

Introduction

JEV (the genus *Flavivirus,* the family *Flaviviridae*) belongs to a mosquito-borne flavivirus group consisting of some 66 antigenically-related viruses (Westaway *et al*., 1985). The virus has gained considerable importance as a human pathogen with an increase in frequency of epidemics of viral encephalitis as recorded in some parts of South-east Asian and Western Pacific regions (Vaughn and Hoke, 1992; Monath and Heinz, 1996). Recently, the disease has also emerged in non-Asian regions such as Northern Australia (Hanna *et al.,* 1996, 1999). The situation however, is further complicated in some parts of South-east Asia, particularly in India due to overlapping epidemics of JEV and Dengue virus (DENV) as well as sporadic cases caused by WNV, which have posed serious hazard to public health (Carey and Myers, 1968; George *et al*., 1984). This outcome has complicated to a great extent both the vaccination and host immunologic response as well (Kimura-Kuroda and Yasui, 1988).

The viral envelope gpE is largely responsible for an antigenic cross-reactivity of flaviviruses. MAbs against gpE have been used to delineate both the physical and functional domains of gpE (Peiris *et al*., 1982; Henchal *et al*., 1985; Heinz, 1986). Mapping of antigenic epitopes of gpE JEV, strain 733913 (group 1 strain of Indian origin) employing corresponding MAbs showed existence of 5 domains represented by MAb groups involving Hs, HAI-negative virus specific (NHs), HAI-positive flavivirus cross-reactive (Hx), HAI-negative flavivirus cross-reactive (NHx), and NHA MAbs (Kedarnath *et al.,* 1986; Cecilia *et al*., 1988). The MAbs representing Hs domain are neutralizing

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Abbreviations: AF = ascitic fluid; AST = average survival time; CHIKV = Chikungunya virus; DENV = Dengue virus; gpE = glycoprotein E; HAI = haemagglutination-inhibition; NHA = HAI-negative cross-reactive; NHx = HAI-negative flavivirus cross-reactive; NHs = HAI-negative virus specific; Hx = HAI-positive flavivirus cross-reactive; Hs = HAI-positive virus specific; i.c. = intracerebral(ly); i.p. = intraperitoneal(ly); JEV = Japanese encephalitis virus; MAb(s) = monoclonal antibody(ies); MR = mortality ratio; WNV = West Nile virus

and protective in mice against infections with group 1 JEV strain 733913, whereas MAbs corresponding NHs and Hx domains protected the mice against infections with group II JEV strains that have lost some of the protective epitopes (Gupta *et al*., 2006, 2008). Since the presence of flavivirus cross-reactive antibodies are considered to be responsible for severe DENV infections, hemorrhagic fever and shock, it would be rational to study the effect of two anti-gpE of JEV MAbs corresponding to NHA domain on JEV infection in mice (Halstead, 1973).

The present study was undertaken to analyze the effect of two cross-reactive MAbs, i.e. NHA-1 (neutralizing) and NHA-2 (non-neutralizing) prepared against gpE of JEV strain 733913 on the mice infected with homologous virus.

Materials and Methods

Virus strain and mice. JEV strain 733913, isolated from a fatal case of Japanese encephalitis from Bankura, India in 1973 was employed. It was maintained by i.c. inoculation in 2-days-old suckling mice and the stock virus was stored at -70°C. Albino mice (Webster or Swiss strain) 3–4 week-old were employed as approved by the Institutional Ethical Committee.

Monoclonal antibodies. MAbs A panel prepared against gpE of JEV strain 733913 in our laboratory were classified as Hs (4 MAbs), NHs (2 MAbs), Hx (5 MAbs), NHx (3 MAbs) and NHA (2 MAbs) according to their reactivity in different assays (Kedarnath *et al.,* 1986; Cecilia *et al.,* 1988). Since the Hs, NHS, Hx, and NHx MAbs worked out for their protective abilities in mice against JEV strain 733913, the two NHA MAbs i.e., NHA-1 and NHA-2 subtype IgG2b and IgM, respectively, were employed in the present study. Both MAbs were HAI-negative and the former neutralized JEV *in vitro* showing a titer of 2.0 (expressed as 1/log10) with 50% inhibition of cytopathic effect. 4 Hs MAbs showed neutralizing titers ranging from 2.95 to 3.69. The two NHA MAbs showed nuclear immunofluorescence in non-infected porcine kidney cells and at the same time recognized JEV antigen in the cytoplasm of infected cells by indirect fluorescent antibody test. Furthermore, both MAbs recognized specifically the antigenic epitopes on JEV gpE that were expressed on the surface of infected cells by a modified indirect fluorescent antibody technique. Moreover, the two NHA MAbs reacted strongly with JEV antigen purified by sucrose density gradient and with histones in ELISA (Gupta *et al.,* 1992a, 1999).

Ascitic fluids (AFs) were obtained from ascites produced in pristane-primed BALB/c mice as described previously. MAb AF prepared against Chikungunya virus (CHIKV) (the genus *Alphavirus*, the family *Togaviridae*) that did not cross-react with JEV gpE, served as a negative control (Gupta *et al*., 2003, 2006, 2008).

Passive immunizations. Groups of about 3 to 4-week-old Swiss mice were inoculated i.c. with 100 LD_{50} of the virus. At 2 days post infection, the mice were i.p. injected with 0.1 ml of immune AF (diluted 1:2) containing the tested or control MAb as described previously (Gupta *et al*., 2003, 2008). The inoculated mice were observed for 21 days; mortality ratio (MR) and average survival time (AST) were calculated and compared with MR and AST of control mice. The brains of few mice harvested just prior to death were assayed for the virus contents in 2 days-old infant mice by the i.c. route and the titers were expressed as log LD_{50} of the virus. Also an attempt was made to detect the virus similarly in the blood samples of these mice.

In another series of experiment, about 3 to 4-week-old Swiss mice were i.p. administered 0.1 ml of immune AF (diluted 1:2) or control AF 1 day prior to the virus challenge (100 LD_{50} of the virus injected i.c.) and MR and AST were calculated (Gupta *et al*., 2003, 2008). The brains and blood samples of few sick mice were assayed for the virus contents similarly as detailed above.

Statistics. Statistical significance of differences were evaluated for experimental mice from control values of MR, if any, by *Chi-square* test and for AST either by *Student's* test or by the non-parametric method of Wilcoxon. p values <0.05 were considered significant.

Results

The effect of Hs MAbs on homologous JEV strain 733913 has already been examined in mice and the results were similar to those obtained in the present study (Gupta *et al.,* 2003). The Hs-1 (MR 10/20 50%, AST 12.4 days) and Hs-4 (MR 7/20 35%, AST 15.11 days) MAbs protected the mice, when given 1 day before challenge with JEV strain 733913 (controls MR 20/20 100%, AST 5.9 days). However, the protection was significantly reduced when Hs MAbs were administered 2 days after the virus challenge. The MAbs Hs-1 (MR 13/20 65%, AST 9.69 days) and Hs-4 (MR 18/20 90%, AST 6.73 days) exerted only a little protection (controls MR 20/20 100%, AST 6.1 days).

In contrast, the mice administered MAbs NHA-1 or NHA-2 either 1 day before or 2 days after the virus challenge failed to protect mice, which showed 100% mortality (MR 20/20). Interestingly, the mice given MAb NHA-1 one day before the virus challenge showed rather reduced survival (AST 4.03 days), which was significant (p <0.005) as compared to control mice (AST 5.73 days) (Table 1). In contrast, no significant differences in the survival of experimental mice (AST 6.13 days) and control mice group (AST 5.9 days) were recorded, when the MAb was administered 2 days after the virus challenge. However, the other MAb (NHA-2) administered either 1 day before or 2 days after the virus challenge showed no reduction in mice survival (AST 5.4 to 5.8 days) in comparison to the control mice (AST 5.73 to 5.8 days).

Assaying of the virus content in the brains of a few mice harvested just prior to death from the experimental and control groups yielded similar virus titers ranging from 4.3 to 6.7 log LD_{50}/0.02 ml, but their blood samples were negative for the virus.

Table 1. AST of mice administered with MAbs before or after challenge with JEV

MAbs	Ig subclass	AST	Statistical significance (*p*)	Virus titers in brains (log LD_{50}/0.02 ml)
MAbs administered 1 day before the infection				
NHA-1	IgG2b	4.03 ± 0.29	<0.005	4.3 to 6.7
Controls	IgG2b	5.73 ± 0.46		5.8 to 6.3
NHA-2	IgM	5.76 ± 0.43	N.S.	4.6 to 6.6
Controls	IgG2b	5.80 ± 0.37		5.1 to 6.6
Hs-1	IgG1	12.40 ± 0.29	<0.001	n.d.
Hs-4	IgG2a	15.11 ± 0.18	<0.001	n.d.
Controls	IgG2b	5.90 ± 0.49		n.d.
MAbs administered 2 days after the infection				
NHA-1	IgG2b	6.13 ± 0.44	N.S.	4.8 to 6.3
Controls	IgG2b	5.90 ± 0.47		5.3 to 6.7
NHA-2	IgM	5.40 ± 0.43	N.S.	5.4 to 6.6
Controls	IgG2b	5.80 ± 0.47		4.6 to 6.7
Hs-1	IgG1	9.69 ± 0.16	<0.05	n.d.
Hs-4	IgG2a	6.73 ± 0.41	N.S.	n.d.
Controls	IgG2b	6.10 ± 0.38		n.d.

n.d. = not done; N.S. = not significant; LD_{50} = lethal dose 50, yielding death in 50% of laboratory animals.

Discussion

Previously, antibody-dependent enhancement of virus infectivity has been recorded in humans and in experimental animals in the infections of Venezuelean encephalomyelitis virus, Langat, DENV, rabies or Feline infectious peritonitis viruses (Berge *et al*., 1961; Webb *et al*., 1968; Sikes *et al*., 1971; Halstead *et al*., 1973; Blancou *et al*., 1980; Prabhakar and Nathanson, 1981; Weiss and Scott, 1981; Scott *et al*., 1983; Gould *et al*., 1987; Henchal *et al*., 1988). The enhancement of virulence in mice has also been reported with various strains of Yellow fever virus and with JEV, strain Beijing and it was induced by two anti-gpE MAbs of Yellow fever virus that showed poor neutralizing ability (Barrett and Gould, 1986; Gould and Buckley, 1989). In the present study, the mice administered with anti-gpE MAb NHA-1 one day before the virus infection, showed early death by about 2 days in comparison to the control mice given control SP2/0 or anti-CHIKV MAb. The present finding is significant and at the same time in contrast to the previous findings, where MAbs Hs, NHs, and Hx administered 1 day before the infection resulted in protection against the lethal virus challenge. However, the protection was minimal, when the MAbs were given 2 days after the infection (Gupta *et al*., 2003, 2008). This indicates a modification of the virus biological activity by MAb NHA-I leading to the enhanced virulence of infecting virus as similar virus titers/yields were recorded in the brains of a few mice harvested just prior to death from the group of control and infected animals (Gould *et al*., 1987; Gould and Buckley, 1989). Examination of viral antigenicity along with the histological changes in brain of infected mice at different time intervals after virus infection remains to be determined. It might reveal the kinetics of viral replication in relation to the early death seen in mice administered with NHA-1 MAb one day before the infection.

Our earlier studies employed i.p. administration of Hs MAbs 1 day prior to or 2 days after i.c. inoculation of JEV resulted in protection of the immunized mice possibly by permitting the antibodies to cross and interact/neutralize the virus inside the brain (Gupta *et al*., 2003, 2008). Although a similar route of MAb administration and virus challenge was employed in the present study, a direct interaction between the virus and NHA-1 MAb remains to be determined. Gould and Buckley (1989) reported a direct interaction between JEV and the MAb that induced early death of mice. Also, no difference in viremia was recorded in nude athymic and euthymic BL6 mice except that in the athymic mice that are lacking antibody production, a second viremic phase of much lower magnitude was produced, when the animals were sick (Lad *et al*., 1993, 2005). Kimura-Kuroda and Yasui (1988) reported similar viremia till day 3 post infection in the mice administered either of the two anti-gpE JEV MAbs, which produced early death by 1 to 2 days as compared to the controls. In both groups the viremia was terminated by day 4 post infection indicating that it had no role in early death of mice (Kimura-Kuroda and Yasui, 1988).

In the present study, early death of the mice administered with MAb NHA-1 one day before infection did not seem to be related to the direct effect produced by MAb. Of the two MAbs that recognized an epitope on histones, one MAb was neutralizing (NHA-1) and produced early death of mice, whereas the other non-neutralizing MAb (NHA-2) did not present the same effect. These MAbs induced conformation-dependent changes in the viral epitopes and therefore, the role of such conformational changes induced by pre-existing MAb NHA-1 in enhancing viral virulence in infected mice can not be ruled out (Gupta *et al.,* 1992a, 1999).

Gould *et al.* (1983) reported the preparation of MAb against protein P74 of JEV that was also reactive with nuclear antigen of Vero cells. Also, antibodies against DENV non-structural proteins were shown to increase DENV mortality in mice (Henchal *et al*., 1988). The exact nature of the epitope on gpE corresponding to NHA-1 MAb remains to be elucidated, although some homology was reported between JEV gpE and calf thymus types 2A and 4 using ALIGN program (Gupta *et al*., 1992a). Furthermore, anti-histone antibodies in Japanese encephalitis patients' sera and presence of both NHA-1 and NHA-2 epitopes were reported on some of the JEV strains tested in our laboratory (Gupta *et al.,* 1992a, b). The presence of cross-reactive antibodies induced by JEV vaccination or infection may represent the risk factor particularly in areas suffering with flaviviruses epidemics and therefore, the present findings may gain much importance (Carey and Myers, 1968; George *et al*., 1984; Kimura-Kuroda and Yasui, 1988).

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