IMMUNE RESPONSE IN MICE INFECTED WITH THE ATTENUATED JAPANESE ENCEPHALITIS VACCINE STRAIN SA14-14-2

T. LEE, T. KOMIYA, K. WATANABE, C. AIZAWA, H. HASHIMOTO

Research Center for Biologicals, The Kitasato Institute, 6-111, Arai Kitamoto-shi, Saitama 364, Japan

Received April 2, 1995

Summary. – We characterized protective immune response in mice infected with the attenuated Japanese encephalitis virus (JEV) strain SA(A) derived from the live JE vaccine strain SA14-14-2. In the sera of mice infected with SA(A), antibodies to JEV envelope (E) and non-structural NS1 proteins were detected by Western blot analysis and neutralization, haemagglutination-inhibition (HAI), and complement fixation (CF) antibodies were detected by serological tests. Production of antibody to NS1 protein strongly indicated peripheral growth of SA(A) in mice and its growth induced the immune response. A single immunization with SA(A) significantly protected mice against lethal intracerebral (ic) challenge with the virulent SA(V) strain.

Key words: Japanese encephalitis virus; attenuated vaccine strain; virus growth *in vivo*; protective immune response; anti-JEV E antibody; anti-JEV NS1 antibody

JEV, a member of the family *Flaviviridae,* is an important cause of mosquito-borne encephalitis. In the People's Republic of China, the live attenuated JE vaccine strain SA14-14-2 was developed from the virulent SA14 strain (Ao *et al.,* 1983; Yu *et al.,* 1988). The live vaccine strain SA14-14-2 was markedly reduced neurovirulence in mice in comparison with the parental strain SA14 (Hase *et al.,* 1993). In our previous study, we compared the nucleotide sequences of SA(V) and SA(A) strains derived from parental strains SA14 and SA14-14-2, respectively, to analyze the molecular basis of the virulence or attenuation (Aihara *et al.,* 1991). In addition, we showed differences in haemagglutination activity and electrophoretic mobility of E protein between SA(V) and SA(A) strains (Lee *et al.,* 1995). These properties of SA(A) E protein appear to be derived from eight amino acid changes in the E coding region. In this study, we examined protective immune response in mice infected with SA(A). It showed that the peripheral growth of SA(A) induced a production of both anti-E and anti-NS1 antibodies, and the antibodies could significantly protect mice against SA(V) challenge.

BHK-21 cells were grown at 37 °C in Eagle's Minimum Essential Medium (MEM) supplemented with 5% foetal calf serum (FCS) and 0.3% tryptose phosphate broth (TPB). Mosquito C6/36 cells were grown at 28 °C in MEM supplemented with 10% FCS and non-essential amino acids (Igarashi, 1978). JEV SA14 and SA14-14-2 strains were obtained from Dr. Yu Yong-Xin. Both the SA14 and SA14-14-2 strains were plaque-purified in BHK-21 cells and then passaged three times in C6/36 cells. The strains derived from SA14 and SA14-14-2 were designated SA(V) and SA(A), respectively.

Four week-old mice (ICR strain) were inoculated intraperitoneally (ip) with 10^4 PFU of SA(V) or SA(A) strains diluted in Hanks' balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA). Brains, spleens, or bloods were obtained from mice after virus inoculation. Brains and spleens were homogenized (10% w/v) with HBSS, centrifuged, and the supernatants were collected. These samples were assayed for virus infectivity by plaque test in BHK-21 cells.

Abbreviations: BSA = bovine serum albumin; CF = complement fixation; FCS = foetal calf serum; HAI = haemagglutination-inhibition; HBSS = Hanks' balanced salt solution; ic = intracerebral(ly); ip = intraperitoneal(ly); JE = Japanese encephalitis; JEV = Japanese encephalitis virus; MEM = Minimum Essential Medium; PAGE = polyacrylamide gel electrophoresis; p.i. = post inoculation; TPB = tryptose phosphate broth

Pooled sera taken from mice 14 days post ip inoculation with SA(A) were subjected to Western blot analysis. Media from SA(V) or mock-infected BHK-21 cell cultures were concentrated by ultrafiltration and mixed with Laemmli sample buffer (62.5 mmol/l Tris-HCl pH 6.8, 3% SDS, 1% 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue). The preparations were electrophoresed on SDS-15% PAGE (Laemmli, 1970) and blotted onto nitrocellulose membranes (Towbin *et al.*, 1979). The latter were incubated with the sera diluted 1:20, then with biotinylated anti-mouse immunoglobulin G (Vectastain) and finally with Vectastain ABC reagent. The membranes were stained with diaminobenzidine and hydrogen peroxide.

Sera individually obtained from mice 14 days post ip inoculation with SA(A) were heat-inactivated for 30 mins at 56 °C and were analyzed by serological tests. Neutralization test of sera was done by 50% plaque reduction of neutralization with SA(V) in BHK-21 cells.

HAI and CF tests of sera were performed using SA(V) antigens prepared from SA(V)-infected mouse brains by sucrose-acetone extraction according to the methods published by Clarke and Casals (1958).

Mouse protection test was performed as follows. Mice were immunized by ip inoculation with 10^4 PFU of SA(A) or with HBSS as a control. At 2 weeks post immunization, the mice were challenged by ic inoculation with 100 LD_{50} of SA(V) and observed daily for 21 days for morbidity and mortality.

We examined virus growth of SA(V) and SA(A) strains in mice after ip inoculation. Table 1 shows virus infectivity in the sera, spleens, and brains. SA(V) was detected in the sera on days 1 and 3 and in the spleens on days 1,3, and 5, with mean peak titers of $10^{3.2}$ PFU/ml and $10^{4.3}$ PFU/g, respectively. SA(V) in the brains appeared on day 3, reached high titers and most of the mice died of encephalitis. On the other hand, SA(A) was detected in the spleens of only two mice on day 3 and one mouse on day 5, and the virus titers were extremely low ($10^{1.7}$ PFU/g). SA(A) was not detected in the sera and brains of all ten mice. SA(A) growth *in vivo* was markedly reduced in contrast to SA(V), and SA(A) showed a lack of viremia and neuroinvasion.

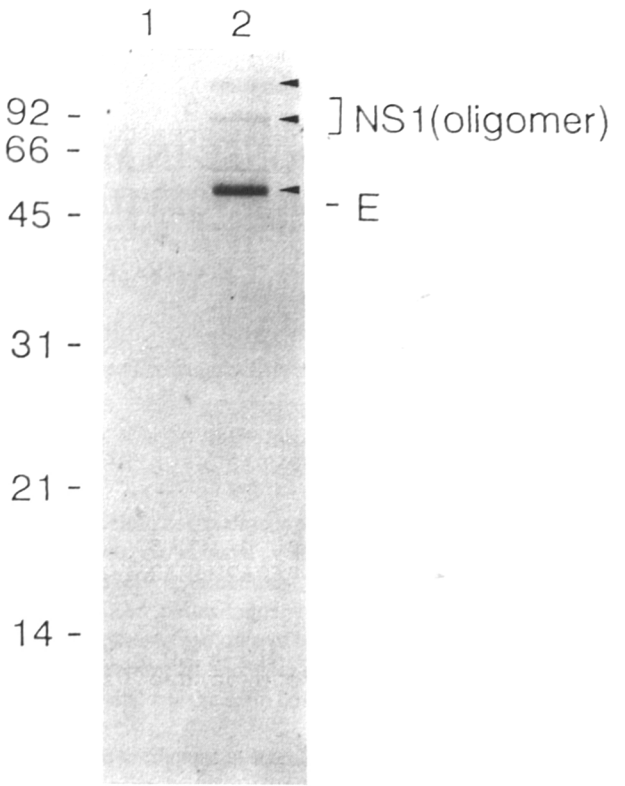

Fig. 1
Western blot analysis of the mouse sera after ip inoculation with JEV strain SA(A)
Mock- and SA(V)-infected BHK-21 cell culture supernatants (lanes 1 and 2, respectively) were resolved by SDS-PAGE and probed with the sera obtained from mice 14 days post ip inoculation with 10^4 PFU of SA(A). E and NS1 (oligomer) proteins, and molecular size markers (M_r x 10^{-3}) are indicated.

We examined antibody response in mice infected with SA(A). The sera of mice taken 14 days p.i. were subjected to Western blot analysis to determine their antibodies spe-

Table 1. Growth of JEV strains SA(V) and SA(A) in mice after ip inoculation

Strain	Organ	Virus infectivity titer (infectivity rate) at various days p.i.			
		1	3	5	7
SA(V)	Serum	2.73 (9/10)	3.24 (7/10)	ND	ND
	Spleen	3.00 (2/10)	4.02 (10/10)	4.29 (9/10)	ND
	Brain	ND	4.18 (4/10)	4.81 (8/10)	6.42 (4/8)
SA(A)	Serum	ND	ND	ND	ND
	Spleen	ND	1.70 (2/10)	1.70 (1/10)	ND
	Brain	ND	ND	ND	ND

Virus infectivity expressed in mean log PFU per g (tissue) or per ml (serum). Infectivity rate = No. of virus-positive mice/No. of mice tested. ND = virus not detected, i.e. virus infectivity titer below 1.00.

Table 2. Antibody response and protection of mice immunized with JEV strain SA(A) against challenge with JEV strain SA(V)

Inoculum	Antibody titers against SA(V)			Mouse protection
	Neutralization test	HAI test	CF test	No. of survivors/No. of tested
SA(A) strain	60.0 ± 11.0	22.2 ± 2.2	11.2 ± 3.2	18/20
HBSS (control)	<10	<10	<4	0/20

Four week-old mice were inoculated ip with the SA(A) strain, followed 14 days later by lethalic challenge with the SA(V) strain. The sera were obtained from mice 14 days p.i. with the SA(A) strain and serum antibodies were tested against the SA(V) antigen. Antibody titers are means ± SE of sera from 10 mice.

cific to JEV proteins (Fig. 1). Antibodies in the sera formed three bands of relative molecular mass of 52 K, 80 K and 100 K. The distinct 52 K band corresponded to JEV structural protein E, and two bands of 80 K and 100 K to JEV non-structural NS1 oligomers (Winkler *et al.*, 1988; Lee *et al.*, 1991). Immunization with SA(A) induced the production of both anti-E and anti-NS1 antibodies in mice. The production of anti-NS1 antibody strongly indicates peripheral growth of SA(A) in mice, since NS1 is a non-structural protein and is not supposed to be contained in SA(A) virus sample inoculated into mice (Mason, 1989).

Neutralization, HAI, and CF antibodies of the sera were then analyzed. They were detected in the sera of all (100%) ten mice and their mean titers were 60.0, 22.2 and 11.2, respectively (Table 2). The results of production of neutralization and CF antibodies induced by SA(A) were similar to those observed for live yellow fever 17D vaccine by Schlesinger *et al.* (1985).

The protective efficacy of immunization with SA(A) was tested in mice against a lethal ic challenge with SA(V) (Table 2). None of the control mice inoculated with HBSS survived the challenge. In contrast, eighteen of twenty (90%) mice immunized with SA(A) survived the challenge with no signs of encephalitis. A single ip inoculation of SA(A) induced significant protection in mice against a lethal ic challenge with virulent strain SA(V). It has been demonstrated that flavivirus E protein induces the production of neutralization and HAI antibodies, and confers a protective immunity in mice (Russell *et al.*, 1980; Gollins and Porterfield, 1986). It is likely that the anti-E antibody possessing neutralization and HAI activities induced by SA(A) is mainly responsible for the protection. It was recently reported that immunization with flavivirus NS1 induced production of CF antibody and protected mice against flavivirus (Gibson *et al.*, 1988). Therefore, anti-NS1 antibody possessing CF activity induced by SA(A) may also be responsible for the protection.

Acknowledgements. We thank Miss R. Uetake for excellent technical assistance.

References

Aihara S, Rao C, Yu YX, Lee T, Watanabe K, Komiya T, Sumiyoshi H, Hashimoto H, Nomoto A (1991): Identification of mutations that occurred on the genome of Japanese encephalitis virus during the attenuation process. *Virus Genes* **5**, 95–109.

Ao J, Yu YX, Tang YS, Cui BC, Jia DJ, Li HM (1983): Selection of a better immunogenic and highly attenuated live vaccine virus strain of Japanese encephalitis. II. Safety and immunogenicity of live vaccine SA14-14-2 observed in inoculated children. *Chin. J. Microbiol. Immunol.* **3**, 245–248.

Clarke DH, Casals J (1958): Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**, 561–573.

Gibson CA, Schlesinger JJ, Barrett ADT (1988): Prospects for a virus non-structural protein as a subunit vaccine. *Vaccine* **6**, 7–9.

Gollins SW, Porterfield JS (1986): A new mechanism for the neutralization of enveloped viruses by antiviral antibody. *Nature (London)* **321**, 244–246.

Hase T, Dubois DR, Summers PL, Downs MB, Ussery MA (1993): Comparison of replication rates and pathogenicities between the SA14 parent and SA14-14-2 vaccine strains of Japanese encephalitis virus in mouse brain neurons. *Arch. Virol.* **130**, 131–143.

Igarashi A (1978): Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J. Gen. Virol.* **40**, 531–544.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee T, Watanabe K, Aizawa C, Nomoto A, Hashimoto H (1991): Preparation of Japanese encephalitis virus nonstructural protein NS1 obtained from culture fluid of JEV-infected Vero cells. *Arch. Virol.* **116**, 253–260.

Lee T, Komiya T, Watanabe K, Aizawa C, Hashimoto H (1995): Differences in haemagglutination activity and electrophoretic mobility of E protein between the parent SA14 and attenuated vaccine SA14-14-2 strains of Japanese encephalitis virus. *Acta Virol.* **39**, 109–112.

Mason PW (1989): Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* **169**, 354–364.

Russell PK, Brandt WE, Dalrymple JM (1980): Chemical and antigenic structure of flaviviruses. In Schlesinger RW (Ed.): *The Togaviruses.* Academic Press, Inc., New York, pp. 503.

Schlesinger JJ, Brandriss MW, Walsh EE (1985): Protection against 17D yellow fever in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. *J. Immunol.* **135**, 2805–2809.

Towbin H, Staehelin BL, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4353.

Yu YX, Zhang GM, Guo YP, Ao J, Li HM (1988): Safety of a live attenuated Japanese encephalitis virus vaccine (SA14-14-2) for children. *Am. J. Trop. Med. Hyg.* **39**, 214–217.

Winkler G, Randolph VB, Cleaves GR, Ryan TE, Stollar V (1988): Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology* **162**, 187–196.