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Host Defence Mechanisms Against Japanese Encephalitis Virus Infection in Mice

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SUMMARY

The role of antibody and cell-mediated immunity in the resistance to Japanese encephalitis virus (JEV) infection was studied in adult mice. Passively transferred antibodies obtained up to 2 weeks after primary infection protected the recipient mice against a challenge infection with JEV. Antibody obtained at 4 or 5 weeks failed to protect despite the presence of high titres of neutralizing antibody. Protection was abrogated by pretreatment of the early serum with 2-mercaptoethanol to remove IgM. Similarly, adoptive transfer of immune spleen cells obtained up to 2 weeks after immunization provided protection. The protective effect was abolished by pretreatment of the immune spleen cells with anti-Thy 1.2 antiserum and complement. These findings suggest a role of T lymphocytes and IgM antibody in recovery from JEV infection.

INTRODUCTION

Host defence against viral infection is mediated by a complex mechanism involving various humoral and cellular effector mechanisms which may act alone (Zisman *et al.*, 1971; Camenga *et al.*, 1974; Chaturvedi *et al.*, 1977, 1978; Rager-Zisman & Allison, 1973; Blanden, 1971) or usually in cooperation (Rabinowitz & Adler, 1973; McFarland *et al.*, 1972).

The outbreak of a wide-spread epidemic of Japanese encephalitis in Uttar Pradesh, India during 1978 (Mathur *et al.*, 1982*a*; Chaturvedi *et al.*, 1980) aroused our interest in this problem. The role of the immune response in recovery from infection with Japanese encephalitis virus (JEV) is poorly understood. Haemagglutination-inhibiting (HAI) antibody has been suggested to play a protective role against JEV in mice (Grossberg & Scherer, 1966) and neutralizing antibody in gibbons (Edelman *et al.*, 1973). Mori *et al.* (1970) have shown that thymus-deprived mice have an impaired anti-JEV antibody response. However, our recent studies show that HAI antibodies do not appear to be the major determinant of recovery after primary JEV infection in mice, nor do they prevent subsequent infection. HAI antibodies were produced after primary infection of pregnant mice but JEV nevertheless persisted and was transmitted transplacentally in consecutive pregnancies (Mathur *et al.*, 1982*b*).

The present study was undertaken to investigate the humoral and cell-mediated immune (CMI) responses in mice and their role in recovery from infection.

METHODS

Virus. JEV strain 78668A was used in the form of an infected adult mouse brain suspension as described previously (Mathur *et al.*, 1981, 1982*a*). It produced 100% mortality by day 6 following intracerebral (i.c.) inoculation, while no mortality was seen after infection using the intraperitoneal (i.p.) route.

Infection of mice. Inbred Swiss albino mice 4 to 6 months old were infected by inoculating 0·5 ml of a 20% JEV-infected mouse brain suspension i.p. In the brain virus assay experiment, mice were also inoculated i.c.

Preparation of spleen cells. Mice were inoculated either i.c. or i.p. with 10^2 LD_{50} of JEV and were killed in batches of three or four daily up to day 21. The spleen cells were teased out in chilled Eagle's minimum essential

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medium containing 10% foetal calf serum (Armour Pharmaceutical, Sussex, U.K.) and a single cell suspension was prepared. The final suspension contained 90 to 95% viable nucleated cells as assessed by trypan blue staining.

Leukocyte migration inhibition (LMI) test. The LMI test was done on spleen cells using the technique of David *et al.* (1964) as described in detail by Chaturvedi *et al.* (1978). The tests for each mouse were done in triplicate. The migration pattern of leukocytes was recorded after incubation for 24 h at 37 °C. The percentage migration inhibition was calculated from the area of cell migration in the presence of JEV antigen compared with normal mouse brain suspension, and an inhibition of 20% or more was considered significant.

Antibody plaque-forming cells (PFC). A spleen cell suspension was prepared and cells forming antibody against JEV were counted by localized haemolysis in gel as described in detail elsewhere (Chaturvedi *et al.*, 1977). Only direct PFC were counted. Multiple slides were prepared for each mouse. Mean values with standard deviations have been calculated after deducting background PFC.

Antibody titre. The HAI test was performed by the technique of Clarke & Casals (1958) using the microtitre technique as described previously (Mathur *et al.*, 1982*a*). The sera were treated with kaolin and heat inactivated at 56 °C for 30 min. Freeze-dried JEV antigen (P20778) was provided by The National Institute of Virology, Pune, India. JEV-specific IgM HAI antibody was detected by fractionation of serum on a sucrose density gradient, with or without treatment of the fractions with 2-mercaptoethanol (2-ME) as described previously (Mathur *et al.*, 1982*a*).

Neutralization test. Sera were inactivated at 56 °C for 30 min and neutralization tests carried out in infant mice as described previously (Mathur *et al.*, 1982*a*).

Depletion of T and B cells. The spleen cell suspension was treated with anti-Thy 1.2 antibodies (New England Nuclear) at 4 °C for 1 h, after which fresh guinea-pig serum (1:6 dilution) was added. After incubation at 37 °C for 1 h, the viable cells were counted by dye exclusion (Golub, 1971). After this treatment 41% of the spleen cells became non-viable. To deplete B cells the spleen cell suspension was treated with anti-mouse IgG serum and complement as described above.

Adoptive immunization by spleen cells. Immune spleen cells obtained at different periods after immunization were injected intravenously (i.v; 1×10^8 cells) into recipient mice, which were challenged 24 h later by inoculation of 10^2 LD_{50} JEV i.c. Mice of control groups received spleen cells from non-immune donors.

Passive immunization by serum. Blood was collected from immunized donor mice at weekly intervals up to 5 weeks after the last dose of JEV. The sera diluted 1:10 or 1:100 were injected in doses of 0·5 ml i.p. into groups of normal mice followed 24 h later by 10^2 LD_{50} of JEV i.c. Animals of control groups received serum from non-immune mice.

2-ME treatment of sera before passive immunization. To study the role of IgM antibody in protection against JEV challenge, the sera obtained at 1 to 5 weeks after immunization were treated with 2-ME and were then dialysed to remove 2-ME as described by Svehag & Mandal (1964). Mice of one group were given the 2-ME-treated immune sera i.v., others were given untreated immune sera and the control group received serum from normal mice. Mice were challenged with 10^2 LD_{50} JEV i.c. 24 h later and were observed for mortality.

RESULTS

Development of the cell-mediated immune response

The LMI has been taken as an index of the cell-mediated immune response produced after JEV infection. The results summarized in Fig. 1 show a significant migration inhibition in the presence of specific antigen from day 3 to day 18 of the study. The response was similar by either route of virus inoculation.

Development of antibody plaque-forming cells

To study the immune response of mice to JEV during the early phase of infection antibody PFC were counted in spleen suspensions. When the virus was given i.p. the PFC count increased from $284/2 \times 10^6$ spleen cells on day 1 to a peak of $640/2 \times 10^6$ spleen cells on day 6 and then declined to $355/2 \times 10^6$ spleen cells on day 9. The PFC counts were similar in mice given virus i.c. (Fig. 2).

Production of neutralizing and HAI antibodies after immunization

Table 1 demonstrates the HAI and neutralizing antibody response estimated in mice at different weeks following i.p. inoculation of JEV. High titres of JEV HAI and neutralizing antibody were found in serum by week 5 post-infection. JEV-specific IgM HAI antibody (2-ME-sensitive) was present in serum 1 and 2 weeks after infection. The presence of JEV-specific

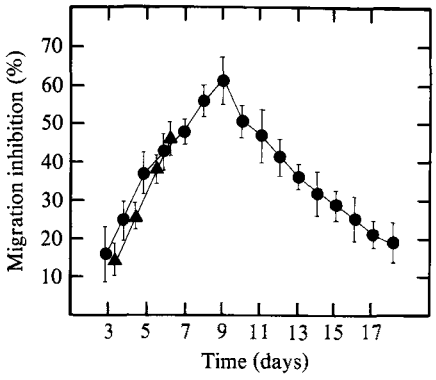

Fig. 1

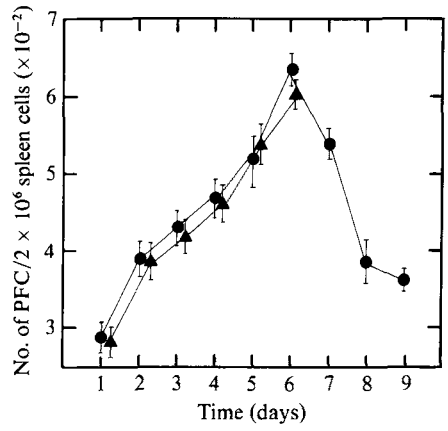

Fig. 2

Fig. 1. Leukocyte migration inhibition of spleen cells of mice on different days after intraperitoneal (●) or intracerebral (▲) inoculation of JEV. Each observation represents the mean value with its standard error from multiple readings from five mice.

Fig. 2. Antibody plaque-forming cells in the spleen of mice on different days after intraperitoneal (●) or intracerebral (▲) inoculation of JEV. Each observation represents the mean value with its standard error from multiple slides from five mice.

Table 1. *Antibody response in mice at different periods after i.p. inoculation of JEV**

Time of post-immunization (weeks)	HAI antibody titre		Neutralizing antibody titre
	Control†	2-ME‡	
1	16	<8	8
2	64	8	32
3	128	64	64
4	128	64	128
5	128	128	256

* Mice were given a 20% JEV-infected mouse brain suspension i.p. Haemagglutinating and neutralizing antibodies were measured at different weeks after virus infection.
† Serum aliquot treated with buffer.
‡ Serum aliquot treated with 2-mercaptoethanol.

IgM was confirmed by screening fractions obtained from sucrose density gradient centrifugation of serum (Table 2).

Assay of virus in brain

The titres of JEV in the brain of mice after i.p. or i.c. inoculation of the virus are presented in Fig. 3. The virus titre gradually increased in mice given virus i.c. but no virus was isolated from the brain of i.p. inoculated mice.

Transfer of resistance by spleen cells

Immune spleen cells obtained from donor mice at 1 and 2 weeks after primary infection were capable of conferring protection to recipient mice against i.c. challenge with JEV (Fig. 4). Spleen cells harvested at 3 to 5 weeks failed to protect the mice. Transfer of normal mouse spleen cells had no antiviral activity. The relationship between the number of transferred immune spleen cells and protection was studied. It was observed that there was little or no protection when less then 10^8 cells were transferred (Table 3).

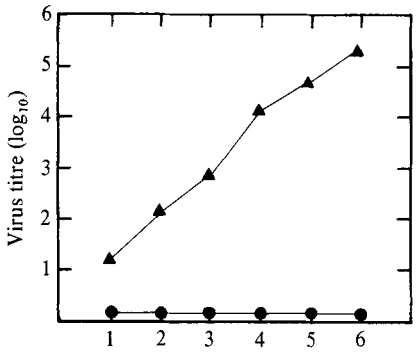

Fig. 3

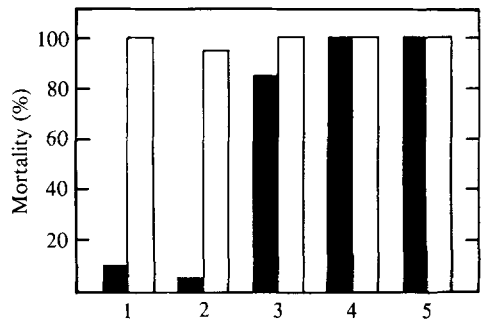

Fig. 4

Fig. 3. Virus titres in brain on various days after intraperitoneal (●) or intracerebral (▲) inoculation of JEV. Each point represents the mean value from five mice.

Fig. 4. Effect of adoptive transfer of immune spleen cells on JEV infection. Spleen cells obtained at 1 to 5 weeks after immunization with JEV were injected intravenously with doses of 10^8 cells followed 24 h later with 10^2 LD_{50} of JEV intracerebrally. ■, Mice given immune spleen cells; □, mice given normal spleen cells.

Table 2. *JEV-specific IgM antibodies in serum fractions of mice**

Time after immunization (weeks)	Serum fraction	HAI antibody titre	
		Control†	2-ME
1	3	4	0
	4	4	0
	5	8	0
2	3	8	0
	4	8	0
	5	16	0
3	3	8	4
	4	16	8
	5	32	32
4	3	16	8
	4	16	16
	5	32	32
5	3	16	16
	4	16	16
	5	32	32

* Mice were given a single i.p. injection of JEV. Blood was collected at weekly intervals. Presence of IgM antibodies in serum fractions obtained by sucrose density gradient centrifugation was detected by radial immunodiffusion (Mancini *et al.*, 1965). IgM was found in serum fractions 3, 4 and 5; therefore, in these fractions HAI antibodies were measured before and after treatment with 2-ME.

† Serum sucrose fraction treated with buffer.

Effect of depletion of T and B lymphocytes on the transfer of resistance by immune spleen cells.

Immune spleen cell suspensions were treated with anti-Thy 1.2 antibody and complement or with anti-mouse globulin and complement. 10^8 cells, either treated or untreated, were injected i.v. into recipient mice, followed by i.c. challenge with JEV. The findings summarized in Table 4 show that the protective effect of the adoptively transferred immune spleen cells is abrogated by depletion of T lymphocytes with anti-Thy 1.2 antibody. Protection was not affected by depletion of B lymphocytes with anti-mouse globulin (Table 4).

Transfer of resistance by antisera

The data presented in Table 5 show that sera obtained at 1 and 2 weeks after primary infection protected the recipients against i.c. challenge with JEV. Sera obtained at 3 weeks after infection provided only slight protection and those from later bleeding none at all.

Table 3. *Relationship between number of immune spleen cells transferred and protection**

No. of spleen cells		Death/total	Mortality (%)
Immune	200×10^6	1/20	5
	100×10^6	1/20	5
	10×10^6	18/20	90
	10^6	19/20	95
Normal	200×10^6	20/20	100

* Immune spleen cells were obtained 7 days after immunization with JEV. Mice receiving i.v. transfers of cells were challenged 24 h later with 10^2 LD_{50} of JEV i.c.

Table 4. *Antiviral activity of immune spleen cells treated with various antisera*

Treatment of immune spleen cells	Time after primary infection (weeks)*									
	1		2		3		4		5	
	% M	MDD	% M	MDD	% M	MDD	% M	MDD	% M	MDD
Immune										
ATS†	95	6	95	6·2	100	6·1	100	6·1	100	6·2
AGS‡	10	11·0	10	11·5	85	8·0	100	7·0	100	6·5
Untreated	10	11·4	5	12·0	85	8·3	95	7·4	100	6·5
Normal	95	6·5	100	6·0	100	6·1	100	6·2	95	6·0
Without cells	100	6·0	100	6·0	100	6·2	100	6·0	100	6·0

* Immune spleen cells were obtained at weekly intervals after a single i.p. dose of 0·5 ml of a 20% JEV-infected mouse brain suspension. 10^8 untreated or pretreated immune spleen cells were injected i.v. into recipient mice which were challenged 24 h later with 10^2 LD_{50} of JEV i.c. % M, Percentage mortality; MDD, median day of death.

† Immune spleen cells treated with anti-Thy 1.2 antiserum and complement.

‡ Immune spleen cells treated with anti-mouse globulin antiserum and complement.

Table 5. *Antiviral activity of immune mouse sera*

Serum dilution	Time after primary infection (weeks)*									
	1		2		3		4		5	
	% M	MDD	% M	MDD	% M	MDD	% M	MDD	% M	MDD
Immune										
1:10	10	12·0	0	0	75	11·4	100	6·5	100	6·0
1:100	20	12·1	5	12·0	85	10·3	100	6·2	100	6·0
Normal										
1:10	100	6·2	100	6·0	95	6·0	95	6·0	100	6·0
Without serum										
0	100	6·0	100	6·1	100	6·7	100	6·3	100	6·2

* Mice were immunized by a single i.p. injection of JEV. Blood was collected at weekly intervals and 0·5 ml serum dilutions were injected i.v. into normal mice which were challenged 24 h later with 10^2 LD_{50} of JEV i.c. A control group was given normal mouse serum. Each group consisted of 20 mice. % M, Percentage mortality; MDD, median day of death.

Passive immunization using 2-ME-treated antisera

The purpose of this experiment was to determine the role of IgM antibodies in conferring protection. Immune sera at different weeks after primary infection were treated with 2-ME to remove IgM antibodies. The mortality was higher in mice given 2-ME-treated antisera than in animals which had received untreated immune sera (Table 6). The difference between untreated and 2-ME-treated serum obtained at week 1 and week 2 after immunization was highly significant ($P < 0{\cdot}001$).

DISCUSSION

In the present study the immune response and the protective effect of passive adoptive immunization were investigated. The leukocyte migration inhibition test showed a short-lived

Table 6. *Failure of 2-mercaptoethanol-treated immune sera to protect against i.c. challenge with JEV**

Time after primary infection (weeks)	2-ME-treated immune serum		Untreated immune serum (1:10)		Normal serum (1:10)	
	% M	MDD	% M	MDD	% M	MDD
1	90	9·0	20	11·5	100	6
2	95	10·0	15	10·2	95	6
3	95	8·2	85	6·5	95	6
4	100	6·0	100	6·0	100	6
5	100	6·0	100	6·0	100	6

* Mice were given 0·5 ml pretreated or untreated sera i.v. and were challenged 24 h later with 10^2 LD_{50} of JEV i.c. Each group consisted of 20 mice. % M, Percentage mortality; MDD, median day of death.

CMI lasting up to 2 weeks after priming. Transient CMI has been shown in a number of studies, namely influenza (Hellman *et al.*, 1972), Sindbis and VEE (Rabinowitz & Adler, 1973) virus infections. The mechanism of such a transient response is not well understood. On the other hand, the humoral immune response to JEV appeared prolonged. The PFC response appeared as the usual 'bell-shaped' curve with peak counts on the 6th day, whereas the HAI and the neutralizing antibodies were first detectable at 1 week and persisted for 5 weeks after infection. The CMI and the humoral immune responses were similar irrespective of whether the virus was given by the i.p. or the i.c. route. Virus given i.c. replicated in the brain reaching high titres while it failed to enter the brain when given by the i.p. route (Fig. 3). Involvement of the brain appears essential to bring about morbidity and mortality in JEV infection.

Adoptive transfer of immune spleen cells had a protective effect against JEV challenge. The protection was abrogated by pretreatment of the immune spleen cells with anti-Thy 1.2 antiserum and complement; pretreatment with anti-globulin antiserum and complement had no effect. This implies that the protection is mediated by T lymphocytes. The immune spleens cells were effective only up to 2 weeks after priming; cells taken at later periods after immunization failed to provide protection. This correlates with the transient presence of CMI as shown by the LMI test. Passive transfer of immune serum obtained up to 2 weeks after priming also provided protection against JEV. The protective effect was abolished by pretreatment of the immune sera with 2-ME. This suggests that the protection against JEV was provided by IgM antibodies; purified specific IgM is required to further confirm this conclusion. Serum obtained after 4 weeks of immunization had high HAI antibody titres but failed to provide protection.

The role of antibodies in protection against JEV infection is not clear. Fatal encephalitis is seen in horses and pigs in the presence of neutralizing and complement-fixing antibodies (Rhodes & van Rooyen, 1968; Ueba *et al.*, 1972). The co-existence of JEV with its HAI antibodies is seen in blood of vaccinated pigs (Ueba *et al.*, 1972). We have observed earlier that JEV is transmitted to the foetus even when the mother had JEV HAI and neutralizing antibodies (Mathur *et al.*, 1982*b*). The HAI and neutralizing antibodies against JEV in man are both IgM and IgG in nature (Ishii *et al.*, 1968). The findings of the present study indicate that IgM antibodies are protective in JEV infection of mice. This observation is supported by the clinical studies of Edelman *et al.* (1976) who reported disappearance of the neurological signs in patients of Japanese encephalitis in the presence of IgM antibodies.

The half-life of IgM antibodies in mice is less than 4 days (Mims, 1983) and the above transfer experiments show that immune cells are effective for 2 weeks only. If JEV escapes immune surveillance during this period, a persistent infection may result. Our earlier studies have shown that when JEV is inoculated i.p. into pregnant mice, infectious virus can be demonstrated in body tissues up to 17 weeks after infection. We were unable to isolate JEV from these animals later; during a subsequent pregnancy, however, virus transmission to the foetus was noted (Mathur *et al.*, 1982*b*). Pregnancy is known to depress the immune response (Ceppellini, 1971). The above experiments show that in the absence of IgM antibodies JEV is not eliminated. Boosting and sustaining the production of IgM antibodies against the virus may be considered as the main host defence mechanism.

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