Cross-Protection Between Group B Arboviruses: Resistance in Mice to Japanese B Encephalitis and St. Louis Encephalitis Viruses Induced by Dengue Virus Immunization

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Albino Swiss mice, immunized with any of several types and strains of dengue viruses, were afforded substantial protection against peripheral Japanese B encephalitis or St. Louis encephalitis virus challenge. Dengue-2 (New Guinea "C")-immunized mice showed, 10 and 20 weeks after immunization, undiminished resistance with concomitant cyclophosphamide treatment and virus challenge. Examination of the effects of immunization on Japanese B encephalitis virus pathogenesis, after virus challenge with concomitant cyclophosphamide treatment, indicated that protection was associated with decreased viremia and virtually no virus replication in the brain as compared with controls. These effects could be demonstrated before detection of any neutralizing antibody to the challenge virus. From the applied aspect, the data support the hypothesis, based on epidemiological evidence and experiments in hamsters, that prior exposure of man to dengue viruses can confer some degree of protection against Japanese B encephalitis or St. Louis encephalitis disease.

Cross-protection among group B arboviruses has been described, on numerous occasions, in several laboratory hosts (3, 9, 13, 14, 17-21). Studies on the relationships between the closely related complex of viruses that include Japanese B encephalitis (JBE), St. Louis encephalitis (SLE), Murray Valley encephalitis, and West Nile viruses have primarily used the hamster, because of its susceptibility to West Nile and Murray Valley encephalitis viruses by peripheral inoculation (9, 13, 18, 20). The potential ability of dengue viruses to produce cross-protection against JBE and SLE viruses was not examined in that model, but was examined against West Nile virus (18, 20). Attempts to demonstrate cross-protection between dengue and West Nile viruses in mice were reported as successful by Bond (Ph.D., University of Pittsburgh, Pittsburgh, Pa., 1967) and as unsuccessful by Price and Thind (18). Due to loss of susceptibility of young adult mice to peripherally inoculated SLE, the challenge tests by Bond had to be made before the 4th week of age; therefore, they might have little significance. With epidemiological evidence of possible cross-protection influences of dengue infection on JBE and SLE disease in man (2, 8, 11), this study was undertaken to develop a mouse model in which to test satisfactorily the ability of various types and strains of dengue

viruses to induce an altered host immune status which would confer protection against peripheral challenge of JBE or SLE virus. It was planned to extend the time period of the former, relatively short-term hamster and mouse studies to determine whether the protection was short-lived or of long duration.

MATERIALS AND METHODS

Mice. Pathogen-free albino Swiss mice (Charles River Breeding Laboratories, Hazelton, Mass.) were used in all experiments. Females, 2 to 3 weeks old at the beginning of the immunization procedure, were used in all challenge experiments. Females, 3 to 4 weeks old, or suckling mice, 2 to 3 days old, were used in neutralization tests.

Viruses. Prototype strains of dengue viruses (Table 1), all in their 23rd or 24th mouse brain passage, were used for immunizations. Challenge was with either JBE (Peking strain) or SLE (Pinellus P₁₆ strain) virus, each in its 4th mouse brain passage. All viruses were stored at -70°C as whole suckling mouse brains until just prior to use, when they were prepared as 10% (wt/vol) suspensions in buffered saline (pH 7.4). Dengue virus pools had suckling mouse intracerebral (ic) mean lethal dose titers of $10^{6.2}$ to $10^{7.2}$ per 0.01 ml. The JBE and SLE virus pools yielded weanling mouse ic mean lethal dose titers of $10^{8.3}$ to $10^{8.6}$ per 0.03 ml and $10^{8.2}$ to $10^{8.4}$ per 0.03 ml, respectively.

Immunization. The procedure was the same for all dengue viruses. The first immunizing injection contained a 10% suckling mouse brain suspension of the

appropriate live virus mixed 1:1 (vol/vol) with Freund complete adjuvant, and was given intraperitoneally in a total volume of 0.4 ml. Three subsequent intraperitoneal injections were given at weekly intervals and consisted of 0.2 ml of a 10% suspension alone. Less than three or four immunizations with dengue virus did not afford significant protection against homologous ic dengue challenge (unpublished data). This is probably due to failure of dengue virus to produce even inapparent infection in weanling and adult mice when given by a peripheral route.

Challenge. Mice were challenged with a 0.2-ml subcutaneous injection of a JBE or SLE virus suspension at intervals of 4, 10, or 20 weeks after the last immunizing injection. Controls consisted of unimmunized mice of the same age. Where indicated, to overcome the age-related resistance to peripheral injection, cyclophosphamide (CY) (Cytosan, Meade-Johnson, Evansville, Inc.), in a dosage of 100 mg/kg was given in a single intraperitoneal injection 24 h after challenge, as used by Nathanson and Cole (16). Challenged mice were then observed 21 days for deaths.

Serology. Weanling mouse ic neutralization tests were employed by using serial virus dilutions (10) of JBE or SLE virus on serum pools obtained from samples of immunized mice, just prior to challenge, and from 21-day survivors of the challenge. Tests for dengue-neutralizing antibody on these same sera were done in suckling mice by the same ic method (10). The antibodies measured were expressed as $\log_{10}$ neutralization indexes.

Individual sera collected during a pathogenesis study (see Results) were tested by plaque-reduction in Vero cells. Cells were grown to confluent monolayers in 3-oz (85.05-g) prescription bottles using Eagle basal medium supplemented with 10% inactivated fetal calf

serum, sodium bicarbonate, and antibiotics. Fivefold dilutions of unheated test sera were made in serum-free culture medium and mixed in equal parts with approximately 80 plaque-forming units of JBE virus per 0.2 ml. The mixtures were incubated for 1 h at 37 C. Cultures were inoculated in triplicate with 0.2 ml of a given serum-virus mixture and allowed to adsorb for 1 h at 37 C. The cultures were overlaid with Eagle basal medium containing 10% agamma calf serum, 1% Noble agar, 0.003% neutral red, sodium bicarbonate, and antibiotics. Bottles were incubated for 1 week at 37 C, at which time plaques were counted. The highest serum dilution calculated to neutralize 80% of the plaques was recorded as the titer.

RESULTS

Effect of immunization with various dengue types and strains on protection against JBE or SLE virus challenge. Groups of mice immunized with various dengue strains were challenged 4 weeks after the last immunizing injection with JBE virus, or, in the case of SLE virus, with concomitant CY treatment (Tables 1 and 2). CY was required with SLE virus, as the mice (10 to 11 weeks old at the time of challenge) had developed resistance.

Significant cross-protection was seen in all immunized groups. Protection against SLE virus was apparently resistant to CY treatment. Prechallenge neutralizing antibody responses to the immunizing viruses were variable, but always, with one exception, at a level considered significant (i.e., >1.7 logs). The exception was

TABLE 1. *Effect of immunization by various types and strains of dengue viruses^a on survival and serology after peripheral JBE virus challenge^b 4 weeks after immunization*

Immunizing viruses	Mortality ratio ^c	Mortality (%)	Prechallenge NI ^d		Postchallenge ^e NI	
			Homologous dengue ^f	JBE ^g	Homologous dengue	JBE
DEN-1 (Hawaii)	2/32	6.3	1.9	0.9	2.4	2.9
DEN-2 (New Guinea "C")	0/29	0	1.8	1.1	3.2	2.9
DEN-2 (Trinidad)	3/42	7.1	3.3	0.6	2.7	2.9
DEN-2 (TH-36)	5/45	11.1	2.4	0.2	3.2	2.2
DEN-3 (H-87)	3/49	6.1	2.1	1.1	2.3	3.6
DEN-4 (H-241)	6/46	13.0	1.8	1.1	1.3	2.2
None (Control)	38/60	63.3	— ^h	—	—	3.0

^a A 0.2-ml amount of virus was given intraperitoneally (i.p.) 4 times at 1 week intervals (first injection, 1:1 (vol/vol) with Freund's complete adjuvant); DEN, dengue virus.

^b JBE (Peking) P₄ was used, and a 0.2-ml amount was given subcutaneously (s.c.) (10^{-1} dilution of a virus pool which had a weanling mouse, ic mean lethal dose (LD₅₀) titer of $10^{8.2}/0.03$ ml).

^c Number of deaths/number inoculated.

^d $\log_{10}$ neutralization index.

^e Twenty-one days postchallenge; pooled sera of survivors.

^f Suckling mouse, ic neutralization test.

^g Weanling mouse, ic neutralization test.

^h Not done.

TABLE 2. *Effect of immunization by various types and strains of dengue viruses on survival and serology after peripheral SLE virus challenge^a 4 weeks after immunization (with CY immunosuppression)*

Immunizing viruses	Mortality ratio ^b	Mortality (%)	Prechallenge NI		Postchallenge NI	
			Homologous dengue ^c	SLE ^d	Homologous dengue	SLE
DEN-1 (Hawaii)	17/55	30.9	2.4	1.4	2.1	<1.5
DEN-2 (New Guinea "C")	17/45	37.8	>2.8	1.1	>3.5	2.1
DEN-2 (Trinidad)	29/60	48.3	>2.7	1.1	>2.8	1.5
DEN-2 (TH-36)	11/55	20.0	2.9	1.3	>5.8	1.5
DEN-3 (H-87)	20/52	38.5	2.1	1.8	3.0	<1.5
DEN-4 (H-241)	25/52	48.1	1.4	1.3	3.2	<1.8
None (Control) ^e	80/90	88.9	— ^f	—	—	1.2

^a SLE (Pinellus P₁₅) P₄ was used, and a 0.2-ml amount was given s.c. (10⁻¹ dilution of a virus pool which had a weanling mouse, ic LD₅₀ titer of 10^{8.9}/0.03 ml). NI, neutralization index; DEN, dengue virus.

^b Number of deaths/number inoculated.

^{c, d} Neutralization tests as in Table 1.

^e Combined data of three controls; differences were not statistically significant by the chi-square test.

^f Not done.

noted in one of two groups given dengue-4 (1.4 logs, Table 2). However, neutralization indexes to JBE or SLE virus were never at a level of significance, except in one of two groups given dengue-3 (1.8 logs, Table 2). These pre-existing antibody levels did not correlate with the degree of observed protection. A strong neutralizing antibody response after JBE virus challenge was noted in pools of survivor sera (Table 1). The antibody response to SLE virus noted in survivors of SLE virus challenge (Table 2) was variable but always at a lower level, which was expected with CY treatment.

Duration of cross-protection against JBE or SLE virus challenge. At intervals of 10 and 20 weeks after the last immunizing injection, groups of dengue-2 (New Guinea "C")-immunized mice and controls were challenged, one with JBE and another with SLE virus. This time CY treatment was used in all experiments, because in this age group of mice there was too little susceptibility by peripheral challenge to even JBE virus. Substantial protection to both viruses was observed in immunized groups at

both postimmunization intervals (Tables 3 and 4). The degree of cross-protection against JBE virus challenge remained about the same at both test intervals, whereas that against SLE virus seemed to increase with time. The 21-day postchallenge neutralizing antibody response to JBE virus in survivors was significant. On the other hand, no response was apparent at this interval in survivors of SLE virus challenge. No explanation for this discrepancy is apparent.

Effect of dengue immunization and CY on JBE virus pathogenesis. To gain some insight into possible mechanisms involved in the cross-protection, several variables were examined after JBE virus challenge, including viremia, virus invasion of the brain, and the development of neutralizing antibody.

In one challenge experiment, one group of 52 mice immunized with dengue-2 (New Guinea "C") virus and a group of 64 unimmunized controls were challenged 20 weeks after the last immunizing injection with JBE virus, followed with CY. Another group of 33 control mice was challenged without subsequent CY treatment.

TABLE 3. *Cross-protection afforded by dengue-2 (New Guinea "C") virus immunization against JBE virus challenge at 10 and 20 weeks after immunization (with CY immunosuppression)^a*

Weeks post-immunization	Dengue immunization	Mortality ratio	Mortality (%)	Prechallenge NI		Postchallenge NI	
				Dengue	JBE	Dengue	JBE
10	Yes	7/28	25.0	>3.6	0.3	>2.5	2.5
	No	29/30	96.7				
20	Yes	8/37	21.6	>2.9	1.0	>2.7	2.5
	No	45/49	91.8				

^a All determinations as in Table 1.

TABLE 4. Cross-protection afforded by dengue-2 (New Guinea "C") immunization against SLE virus challenge at 10 and 20 weeks after immunization (with CY immunosuppression)^a

Weeks post-immunization	Dengue immunization	Mortality ratio	Mortality (%)	Prechallenge NI		Postchallenge NI	
				Dengue	SLE	Dengue	SLE
10	Yes	32/55	58.1	2.9	1.3	2.4	<1.5
	No	30/30	100.0				
20	Yes	10/63	15.9	3.1	1.6	2.4	0.6
	No	33/36	91.7				

^a All determinations as in Table 1 except challenge, administered as in Table 2.

At 2-day intervals after challenge, three mice from each group were bled, and their brains were removed. Sera and brains were stored individually at -70 C until tested.

Titers of viremia and virus in the brain were assayed in suckling mice. Individual sera were diluted 1:5 and 1:15 (vol/vol) in normal rabbit serum-sucrose phosphate glutamate (10), and then 0.01 ml of each dilution was inoculated ic. Individual brains were triturated to 10% (wt/vol) suspensions in buffered saline (pH 7.4). Tenfold dilutions of this material were made in the above diluent, and 0.01 ml of each dilution was inoculated ic in each of eight mice. Neutralizing antibody assays were performed by the plaque reduction method.

The course of JBE virus infection differed markedly between the three treatment groups (Tables 5-7). Immunized mice treated with CY showed rather sporadic, low-titered viremias

(Table 5), detectable up to 8 days after challenge. Virus was detected in the brain only once (Table 6), and this was 10 days after challenge. Of note was the detection of a relatively late neutralizing antibody response (Table 7) 10 days after challenge.

Conversely, unimmunized control mice treated with CY had viremias detectable from 2 to 8 days after challenge. Virus was detected in the brain, in varying titers, from 6 to 10 days after challenge. There was no evidence of a neutralizing antibody response during the 10 days of sampling after challenge. The variability of virus titers observed in these brains indicated that the progress of the infection differed in individual mice. This may have been a reflection of the age-related resistance, with the virus having some difficulty establishing itself in neural tissue (1, 16).

Unimmunized control mice, not treated with

TABLE 5. Effect of dengue-2 (New Guinea "C") immunization on viremia in mice challenged with JBE virus^a 20 weeks after immunization (with CY immunosuppression)

Treatment	Mouse no. (day-by-day)	Virus titer in blood ^b (days after challenge)					Mortality (%) ^c (D/I) ^d
		2	4	6	8	10	
Immunized + CY	1	0.9	<0.7 ^e	0 ^f	0.9	0	21.6 (8/37)
	2	0.8	— ^g	<0.7	0	0	
	3	0	<0.7	0.8	0	0	
Unimmunized + CY	1	1.0	>1.2	>1.2	>1.2	0	91.8 (45/49)
	2	<0.7	>1.2	>1.2	>1.2	0	
	3	0.9	>1.2	>1.2	—	0	
Unimmunized, no CY	1	<0.7	0	0	0	0	16.7 (3/18)
	2	<0.7	0	0	0	0	
	3	0	0	0	0	0	

^a JBE (Peking) P₄ was used, and a 0.2-ml amount was given s.c. (10⁻¹ dilution of a virus pool which had a weanling mouse, ic LD₅₀ titer of 10^{8.6}/0.03 ml).

^b Log₁₀ suckling mouse; ic LD₅₀ titer per 0.01 ml of serum.

^c Percent mortality observed in groups from which test mice were sampled.

^d Number of deaths/number inoculated.

^e Less than 50% of suckling mice dying at 1:5 (vol/vol) serum dilution.

^f No detectable virus.

^g Not done.

TABLE 6. *Effect of dengue-2 (New Guinea "C") immunization on invasion of the brain by JBE virus after challenge^a 20 weeks after immunization (with CY immunosuppression)*

Treatment	Mouse no. (day-by-day)	Virus titer in brain ^a (days after challenge)				
		2	4	6	8	10
Immunized + CY	1	0 ^c	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	2.4
Unimmunized + CY	1	0	0	<2.0 ^d	<2.0	3.7
	2	0	0	4.2	4.3	<2.0
	3	0	0	3.5	2.1	<2.0
Unimmunized, no CY	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0

^a JBE (Peking) P₄ was used, and a 0.2-ml amount was given s.c. (10⁻¹ dilution of a virus pool which had a weanling mouse, IC LD₅₀ titer of 10^{8.6}/0.03 ml).

^b Log₁₀ suckling mouse; IC LD₅₀ titer per 0.01 ml of 10% brain suspension.

^c No detectable virus.

^d Less than 50% of suckling mice dying at 10⁻² dilution.

CY, showed only minimal signs of infection. There was low-titered viremia, which was detected in two mice 2 days after challenge (Table 5). No virus was detected in the brains (Table 6), but a neutralizing antibody response was detectable 8 days after challenge (Table 7).

DISCUSSION

Albino Swiss mice, immunized with any of several strains or types of dengue virus, were protected against peripheral JBE or SLE virus challenge. Protection was resistant to CY treatment administered with challenges and, as measured in dengue-2 (New Guinea "C")-immunized mice, remained undiminished for at least 20 weeks after immunization.

The results suggest that the immunizing and challenge viruses share antigens that are involved in the induction of an altered immune status responsible for protection. Protection might then be effected by pre-existing cross-reactive antibody, an anamnestic antibody response elicited by the hypothesized common antigen, or by some facet of a cell-mediated response. Our experiments did not rigorously differentiate between these possibilities; thus, we can only speculate as to their relative importance.

Prechallenge neutralizing antibody levels to challenge viruses were not correlated with levels

of protection. Therefore, it seems unlikely that this was the sole factor involved. However, this facet of the system needs more study, especially since the serological data were derived from serum pools. With this, the degree of individual variability and, thus, the effect of such variability on the outcome of each challenge is not known. Towards this end, passive immunization studies might give more definitive results.

The data from experiments utilizing CY treatment did not support the hypothesis (3, 17) that an anamnestic neutralizing antibody response to the challenge virus, as measured by serum antibody levels, was responsible for protection. Tables 5 and 6 indicate that the critical points of JBE virus pathogenesis (viremia, virus replication in the brain (1)) occur in CY-treated, unimmunized mice about 6 days after infection. These were resolved or prevented in CY-treated, immunized mice well before serum neutralizing antibody was detected (Table 7). If these animals had been developing an anamnestic neutralizing antibody response, it would have been readily detectable in this experiment (Table 7). Lending some support to this, data from survivor serum pools of CY challenge experiments (Tables 2, 3, 4) indicated that protection was not correlated with increases in levels of neutralizing antibody to the challenge virus.

TABLE 7. *Effect of dengue-2 (New Guinea "C") immunization on the development of a neutralizing antibody response to JBE virus after challenge^a 20 weeks after immunization (with CY immunosuppression)*

Treatment	Mouse no. (day-by-day)	Serum dilution neutralizing 80% of JBE plaques ^b (days after challenge)		
		6	8	10
Immunized + CY	1	<1:5	<1:5	1:5
	2	<1:5	<1:5	<1:5
	3	<1:5	<1:5	1:10
Unimmunized + CY	1	<1:5	<1:5	<1:5
	2	<1:5	<1:5	<1:5
	3	<1:5	— ^c	<1:5
Unimmunized, no CY	1	<1:5	1:100	1:80
	2	<1:5	1:5	1:100
	3	<1:5	<1:5	1:8

^a JBE (Peking) P₄ was used, and a 0.2-ml amount was given s.c. (10⁻¹ dilution of a virus pool which had a weanling mouse, ic LD₅₀ titer of 10^{8.6}/0.03 ml).

^b JBE (Peking) P₄ was used; 0.2 ml of a dilution with approximately 80 plaque-forming units/0.2 ml was inoculated onto Vero cells.

^c Not done.

The role of a cell-mediated immune response was not specifically examined in this work. However, the fact that protection was resistant to CY treatment suggests consideration of this possibility. CY treatment delays the initiation of both primary and secondary humoral immune responses (4), but fully differentiated, long-lived lymphocytes and plasma cells escape depletion by short courses of the drug (15). Phagocytic mechanisms involving non-proliferating cells are, likewise, unaffected (4). It is possible that protection may have been effected by these CY-resistant components, acting separately or in conjunction with low levels of specific antibody, not detectable by the methods used here.

More definitive studies will require demonstration of the forementioned effects on pathogenesis in animals still peripherally susceptible, since JBE virus infection, as measured in these normally resistant, CY-treated mice, developed at a much slower rate than observed by Huang and Wong (12) in mice of a susceptible age, inoculated similarly. This suggests that even with CY treatment the virus has difficulty establishing itself. Therefore, it is possible that a partially functional immune mechanism may have sufficed in this artificial situation.

The data support the hypotheses of Hammon (8), Hammon et al. (11), and Bond and Hammon (2), originally based on epidemiological evidence that, in man, prior exposure to dengue confers some degree of protection against JBE and SLE disease. However, the strength of this support rests on the degree to which analogous immune mechanisms might participate. The pathogenesis of JBE and SLE viruses appears to be, at least superficially, similar in both hosts, i.e., hematogenous spread of the virus from extraneural foci to the central nervous system (1, 12, 16). Therefore, it is possible that similar mechanisms might be involved in preventing JBE or SLE disease.

A more difficult problem lies in defining the circumstances under which cross-protection in humans might occur. Serological data from recent studies (5-7) of a JBE epidemic in northern Thailand (a focus of endemic dengue) present evidence tentatively not supporting these hypotheses (5, 6). Grossman et al. (5, 6) concluded that persons with pre-existing group B antibody (presumably dengue) were not guaranteed protection against encephalitis after JBE virus infection. Thus, if the phenomenon of cross-protection between dengue and JBE does, in fact, occur in humans, it is by no means absolute (2, 8, 11) and may require factors in addition to prior infection by dengue viruses.

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