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The mechanism of cross-protection afforded by dengue virus against West Nile virus in hamsters*

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SUMMARY

The protection afforded by similar concentrations of different dengue virus serotypes against a subsequent challenge of West Nile virus was studied in hamsters. The New Guinea C strain of dengue 2 virus gave the best protection. It was found that the anamnestic neutralizing antibody response induced by the challenge West Nile virus against West Nile virus in hamsters, previously immunized with dengue 2 virus, might play a major role in the cross-protection observed in this system.

INTRODUCTION

Studies were recently reported on the protection afforded by a previous injection of dengue virus against a subsequent challenge of West Nile virus in hamsters and on the failure of dengue immune monkeys to develop viremia after challenge with St Louis encephalitis virus (Sather & Hammon, 1968, 1970).

In a recent paper from this laboratory the mechanism of the cross-protection afforded by dengue virus against a subsequent challenge of West Nile virus was studied in hamsters and it was shown that this protective effect was not mediated by interferon or serum protective factor; furthermore, the cross-protection was not due to the mutual exclusion principle (Price & Thind, 1972). The relationship of the protective effect to the *in vivo* production of serum neutralizing antibody was not definitively established. It is the purpose of this paper to report further experiments on the ability of different dengue serotypes to protect hamsters against a subsequent challenge of West Nile virus. Furthermore, the relationship of *in vivo* produced serum neutralizing antibody induced by the challenge virus to the cross-protection was studied.

MATERIALS AND METHODS

Viruses

All virus types used were described in detail in the previous publication (Price & Thind, 1972). All virus pools, as whole infected mouse brains, were stored at −70° C. Freshly prepared homogenized brain suspensions in 0·75 % bovine plasma albumin in phosphate buffered saline, pH 7·4, clarified by centrifugation at 500 *g* for 10 min., were used for vaccination and challenge of animals. All virus

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pools were titrated intracerebrally (i.c.) in 1 to 2-day-old mice. West Nile virus was also titrated subcutaneously (s.c.) in 10- to 11-week-old hamsters when necessary. Unless otherwise stated, LD 50 refer to (s.c.) hamster LD 50.

Cyclosphosphamide

Cyclophosphamide (Mead, Johnson, Evansville, Indiana) was given by the intraperitoneal (i.p.) route in a dose of 60 mg./kg. body weight 24 hr. before and after the challenge virus. There was no mortality associated with this dosage.

Immunization and challenge

Three to 4-week-old hamsters (each weighing 40–60 g.) were immunized by the i.p. route with $10^{7 \cdot 5}$ newborn mouse i.c. LD 50 of the New Guinea C strain of dengue 2 virus unless otherwise specified. Six weeks later the hamsters were challenged subcutaneously with $10^{7 \cdot 5}$ (s.c.) hamster LD 50 of West Nile virus in 0·3 ml. unless otherwise specified. Control hamsters were given 0·3 ml. of a 10% clarified suspension of normal newborn mouse brain. All hamsters were observed for 21 days after challenge.

Serum neutralizing antibody

Neutralization tests for West Nile virus antibody were carried out as described previously using the plaque reduction method (Price & Thind, 1972).

To determine the neutralizing antibody for dengue viruses the same method was used as described previously (Price & Thind, 1972). Twenty-four 1 to 2-day-old mice were inoculated intracerebrally with each virus dilution.

RESULTS

Influence of dengue virus type on protection against subsequent West Nile virus challenge

Previous results (Sather & Hammon, 1968, 1970) indicated that of the four dengue serotypes tested dengue 2 New Guinea C (NGC) isolate gave the best protection in hamsters against a subsequent challenge of West Nile virus. Similar results were later reported from this laboratory (Price & Thind, 1972). However, in our previous experiments the concentration of the various dengue serotypes varied over a tenfold range. Since there was little multiplication of dengue virus under the experimental conditions, the concentration of virus that was inoculated was of critical importance and unfortunately dengue 3 virus and dengue 4 virus were inoculated in a lower concentration than dengue 2 virus. Therefore, this time, very similar concentrations of the various dengue serotypes were used for vaccination. From Table 1 it can be seen that under these conditions dengue 2 New Guinea C isolate still gave the best protection against a subsequent challenge of West Nile virus. Next in order came the other two types of dengue 2 virus and type 1 dengue virus. Types 3 and 4 dengue virus gave the lowest protection.

Table 1. *Protection in hamsters immunized with various types of dengue virus and challenged 6 weeks later with West Nile virus*

Immunizing type	Dose of immunizing type (suckling mouse i.c. LD 50)	Neutralization index to dengue viruses†	Survival*
Dengue 1	$10^{6\cdot5}$	2·1	22/50
Dengue 2 NGC	$10^{6\cdot4}$	2·7	42/50
Dengue 2 NGB	$10^{6\cdot7}$	2·2	27/50
Dengue 2 Trinidad	$10^{6\cdot0}$	1·9	28/50
Dengue 3	$10^{6\cdot2}$	1·7	10/50
Dengue 4	$10^{6\cdot5}$	1·6	7/50
Normal mouse brain	—	0	0/50

* Numerator represents number of hamsters that survived and denominator represents number inoculated. All hamsters were challenged s.c. 6 weeks after immunization with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus. In all tables when hamsters are inoculated with West Nile virus, the LD 50 refers to hamster LD 50 in 0·3 ml. of inoculum.

† Neutralization index is to homologous virus.

Table 2. *Protection and neutralizing antibody titres in hamsters immunized with dengue 2 NGC virus or dengue 4 virus and challenged 6 weeks later with a large concentration of West Nile virus*

Expt. no.	Immunizing inoculum*	Serum dilution which neutralized 50 % of West Nile virus plaques. Days after challenge			Survival in observation group†
		0	4	6	
1	Dengue 2 NGC	0	1/4	1/512	8/10
	Dengue 4	0	1/4	1/128	7/50
	Normal mouse brain	0	1/2	1/128	0/10
2	Dengue 2 NGC	0	1/8	1/1024	9/10
	Normal mouse brain	0	0	1/128	0/10
3	Dengue 2 NGC	0	1/4	1/512	7/10
	Normal mouse brain	0	1/2	1/128	0/10

* $10^{7\cdot5}$ to $10^{7\cdot1}$ suckling mouse i.c. LD 50 of dengue 2 NGC in a volume of 0·3 ml was given i.p. to each immunized hamster. 0·3 ml. of 10% normal mouse brain was given i.p. to each control hamster. All hamsters were challenged s.c. with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus 6 weeks after immunizing inoculum.

† Numerator shows number of hamsters surviving and denominator shows number inoculated.

Protection and serum neutralizing antibody titres

Three separate experiments were carried out to determine the neutralizing antibody titres to West Nile virus after challenging with the large dose of West Nile virus. Samples were stopped after 6 days because many of the control hamsters started to die on the 7th day after challenge. Table 2 shows that in all three experiments the hamsters that were previously immunized with the dengue 2 NGC isolate formed fourfold to eightfold more neutralizing antibody against the challenge virus than the hamsters immunized with normal mouse brain. It will also be noted from Table 2 that very few hamsters immunized with dengue 4 virus survived the challenge with West Nile virus. There is no anamnestic neutralizing

Table 3. *Protection and neutralizing antibody responses of Dengue 2 NGC immunized hamsters* challenged with large and small concentrations of West nile virus*

Expt. no.	Initial treatment	Dose of West Nile challenge virus LD 50 (sc)	Serum dilution which neutralized 50 % of West Nile virus plaques. Days after challenge 0	Days after challenge 6	Survival in observation groups†
1	D_2NGC	$10^{7.5}$	0	1/512	8/10
	NMB‡	$10^{7.5}$	0	1/128	0/10
	D_2NGC	$10^{2.5}$	0	1/128	0/10
	NMB	$10^{2.5}$	0	1/64	1/10
2	D_2NGC	$10^{7.5}$	0	1/512	8/10
	NMB	$10^{7.5}$	0	1/128	0/10
	D_2NGC	$10^{2.5}$	0	1/128	1/10
	NMB	$10^{2.5}$	0	1/32	0/10

* All specified hamsters immunized with dengue 2 NGC strain as described in Table 2. Challenged with varying doses of West Nile viruses 6 weeks after immunization.

† Same as Table 2.

‡ Normal mouse brain suspension.

antibody response against the challenge virus under these latter conditions. Since dengue-immunized hamsters inoculated with $10^{2.5}$ LD 50 (s.c.) of West Nile virus do not survive (Price & Thind, 1972), neutralizing antibody was determined in dengue-immunized hamsters challenged with either $10^{7.5}$ or $10^{2.5}$ LD 50 (s.c.) West Nile virus. Two such experiments were carried out. In both instances the hamsters inoculated with $10^{7.5}$ LD 50 (s.c.) of West Nile virus formed four-fold more neutralizing antibody than the hamsters challenged with $10^{2.5}$ LD 50 (s.c.) of West Nile virus (Table 3). It will be noted that the dengue-immunized hamsters inoculated with the low concentration of West Nile virus formed the same amount of antibody as hamsters immunized with normal mouse brain and challenged with the large dose of West Nile Virus as shown in Table 2, and there was the same survival rate in these both groups.

Effect of cyclophosphamide on cross-protection and neutralizing antibody response

In view of the results shown in Tables 2 and 3 it was thought of interest not only to see the effect of cyclophosphamide on the cross-protection, but to see the effect of the immuno-suppressant drug on the neutralizing antibody response. To do this a group of hamsters were immunized with dengue 2 NGC isolate. Six weeks later they were divided in half. One half received cyclophosphamide and 24 hr. later all hamsters were challenged with $10^{7.5}$ LD 50 (s.c.) of West Nile virus. Twenty-four hours later the hamsters that received the cyclophosphamide received another injection of cyclophosphamide. Table 4 shows that cyclophosphamide completely abolished the cross-protection afforded by dengue virus against a subsequent challenge of West Nile virus. Furthermore, it completely inhibited the neutralizing antibody response induced by West Nile virus against West Nile virus.

Table 4. *Effect of cyclophosphamide administration on cross-protection and neutralizing antibody response against West Nile virus**

Expt. no.	Cyclophosphamide administration	Serum dilution which neutralized 50 % of West Nile plaques. Days after challenge 0	Days after challenge 6	Survival in observation group†
1	No	0	1/512	7/10
	Yes	0	0	0/10
2	No	0	1/1024	8/10
	Yes	0	0	0/10

* All hamsters immunized with dengue 2 NGC strain as described in Table 2 and challenged s.c. 6 weeks later with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus. The cyclophosphamide group received 60 mg. of the drug per kg. 24 hr. before challenge and 24 hr. after challenge.

† Numerator shows number of hamsters surviving while denominator shows number inoculated in the observation groups.

Table 5. *The effect of cyclophosphamide during Dengue immunization on the subsequent anamnestic neutralizing antibody response against the West Nile virus challenge**

Immunizing inoculum	Time of administration of cyclophosphamide	Neutralization index to dengue 2 virus	Serum dilution which neutralized 50 % of West Nile virus plaques. Days of challenge 0	Days of challenge 4	Days of challenge 6	Survival in observation group†
engue 2 NGC	None	2·7	0	1/4	1/512	8/10
engue 2 NGC	During immunization‡	0	0	1/4	1/512	7/10
ormal mouse	None	0	0	1/2	1/64	0/10

All animals challenged s.c. with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus.

Numerator shows number of hamsters surviving while denominator shows number inoculated in the rvation groups.

Cyclophosphamide in a dose of 100 mg./kg. body weight was given 24 hr. before and after immunizing ulum. Three additional doses of cyclophosphamide (100 mg./kg. body weight) were given i.p. 10 days rt after the immunizing inoculum.

It was thought of interest to determine whether an anamnestic neutralizing antibody response to West Nile virus occurred when dengue neutralizing antibody had been suppressed by the use of cyclophosphamide during the immunization period. These experiments were carried out exactly as described previously (Price & Thind, 1972) except that the anamnestic neutralizing antibody response was measured to West Nile virus as well as the cross-protection. Table 5 shows that there is a similar anamnestic response whether cyclophosphamide is administered during the course of immunization or not.

Effect of passive immunization of hamsters on West Nile virus challenge

Since the titres of neutralizing antibody due to the anamnestic response produced by large concentrations of West Nile virus in hamsters sensitized by NGC

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Table 6. *Effect of different amounts of passively administered neutralizing antibody on challenge with West Nile virus*

Group	Level* of passively administered neutralizing antibody at time of challenge	Survivors† Challenged with $10^{7.5}$ LD 50 (s.c.) West Nile virus	Survivors† Challenged with $10^{2.5}$ LD 50 (s.c.) West Nile virus
1	1/512	8/10	9/10
2	1/128	2/10	2/10
3	1/64	0/10	1/10
4	0 (no passive antibody given)	0/10	0/10

* Serum dilution which neutralized 50 % of West Nile virus plaques

† Numerators show number of hamsters surviving and denominators show number challenged with West Nile virus

dengue 2 virus might be related to the protection observed in these hamsters, it was thought of interest to study the effect of different amounts of passively administered neutralizing antibody on West Nile virus challenge. Three groups of hamsters were passively immunized with different amounts of antisera prepared in hamsters. From Table 6 it can be seen that good protection was observed in hamsters having a dilution of 1/512 of neutralizing antibody against West Nile virus challenged with high or low doses of West Nile virus while a fourfold lower dilution of passively administered antibody resulted in little protection. These results would fit in with the hypothesis that the anamnestic neutralizing antibody produced by large concentrations of West Nile virus in previously sensitized dengue 2 NGC hamsters accounts for the protection observed in these animals.

DISCUSSION

The data reported in this paper show that when similar concentrations of different dengue virus serotypes are studied in hamsters, the New Guinea C strain of dengue 2 virus gave the best protection against a subsequent challenge of West Nile virus. This confirms earlier observations (Sather & Hammon, 1970).

The results reported here suggest that in hamsters, in the dengue virus–West Nile virus system, the anamnestic neutralizing antibody response to West Nile virus produced in a hamster previously sensitized with dengue virus and challenged with large doses of West Nile virus plays a key role in the cross-protection described. This hypothesis brings to mind a similar hypothesis made previously concerning cross-protection in other group B arbovirus systems (Price *et al.* 1963) and for cross-protection among group A viruses and between viruses in the Bunyamwera group (Casals, 1963). These experiments may also be related to those which showed that when 'lightly' immunized mice were challenged intracerebrally with varying doses of western equine encephalomyelitis virus death occurred following small but not large virus inocula (Scheslinger, 1949). It was also suggested earlier

that the booster response in a partially immunized animal was associated with resistance to the related virus challenge using various group B arbovirus combinations (Imam & Hammon, 1957). However, it may well be that increased resistance and the higher titres of neutralizing antibody are not directly related but are the concurrent manifestations of an altered immune state.

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