The Growth of Arboviruses in Organ Cultures of Mouse Meninges and the Influence on *in Vitro* Virus Growth of Previous Vaccination (36624)

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The interaction of arboviruses with cells of the central nervous system has not been studied *in vitro*. The successful application of organ culture techniques to a variety of tissues suggested that the meningeal membranes of the central nervous system might also be maintained in organ culture and serve as host cells for virus replication. We report here the replication of arboviruses in murine meningeal organ cultures and the effect of prior vaccination on this replication.

Materials and Methods. Animal tissue. Meninges were obtained from CBA mice following ether anesthesia. The scalp was removed with full aseptic precautions and the skull cap was removed with scissors. In the early experiments the meninges were stripped from the skull. In later experiments the meninges were cultured attached to the skull for easier manipulation. Three to four specimens were obtained for culture from each animal.

Virus pools. West Nile virus pool was a 10% suspension of suckling mouse brain in broth from an original pool kindly supplied by Dr. James Porterfield. Animal vaccination was performed by ip inoculation of $10^3/TCD_{50}$ virus in 0.2 ml.

Yellow fever virus was the 17D strain prepared as above and stored as a 10% brain suspension in broth.

Semliki Forest virus was prepared and stored in the same way.

Culture techniques. The tissue was cultured in Eagle's medium plus 0.2% bovine plasma albumin as used to support the growth of viruses in organ cultures of trachea (1) and alimentary tract (2), in an atmosphere of 5% CO_2 in air at 37°. The tissue was cultured either in roller tubes containing 1.0 ml of medium (3) or static in W.H.O. plastic hemagglutination plates in 0.5 ml of medium.

Procedure. The tissues were cultured for 2 days prior to the onset of the experiments to allow previously absorbed circulating antibody to wash away. The basic experiment consisted of the inoculation of the cultures which were then incubated for 3 hr at 37° to allow virus absorption, and then washed 3 times to remove unabsorbed virus. The cultures were then reincubated, and the medium was changed either daily or on alternate days. Harvested medium was mixed with an equal volume of broth and stored at —70° for later titration using a plaque-assay technique in pig kidney cells (4). In some experiments immune lymphocytes taken from mice which had recovered from West Nile viral infections were added to cultures in plastic plates after the initial 3 hr absorption period.

Three studies were performed: (i) to determine whether meninges in culture would support the growth of a number of arboviruses; (ii) to compare the growth of these viruses in normal and immune meninges; (iii) to determine whether meninges from mice recovered from a virus infection (immune meninges) harbor latent arboviruses which can be induced to replicate.

Results. Study 1. The meninges from healthy adult mice were stripped from the skull and cultured in roller tubes. Three groups of cultures were inoculated with 0.1 ml of virus containing 10^4 TCD_{50}/ml of yellow fever, 10^4 TCD_{50} of Semliki Forest and 10^3 TCD_{50} of West Nile virus, respectively.

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TABLE Ia. Growth of Arboviruses in Organ Cultures of Mouse Meninges.

Virus	Titer of virus (pfu/ml)		Titer of virus (pfu × 10^{-3}/ml) on indicated day after inoculation					
	Inoculated (pfu × 10^{-3}/ml)	After washing	1	2	3	4	6	8
Yellow fever	100	0	0.01	0.1	0.25	2	0.25	0.225
Semliki Forest	100	0	0	—	10	1.5	0.1	0
West Nile (i)	10	0	0.2	0.5	30	0.25	0	0
(ii)	10	0	—	3.5	—	20	1.25	0

TABLE Ib. Comparison of West Nile Virus Growth in Cultures of Meninges Attached and Stripped from Skull.

	Titer of virus (pfu/ml)		Titer of virus (pfu × 10^{-3}/ml) on indicated day after inoculation			
	Inoculated (pfu × 10^{-3}/ml)	After washing	2	4	6	8
Meninges alone	10	0	12	24	50	1.2
Meninges + skull	10	0	2.6	10	34	10

The cultures were incubated, washed and harvested as previously described, and the results are shown in Table Ia. After washing, no virus was detected in the culture medium, but its subsequent appearance and disappearance strongly suggested that growth had occurred in the cultured tissues. Multiplication certainly occurred in the experiments with West Nile virus, as more virus was obtained from the culture than was inoculated. No virus was obtained after 2 days from control dishes inoculated with the cultures containing no tissue but treated identically. In one further experiment, skulls with meninges from 4 mice were split, meninges were stripped from half of the sections, and growth of West Nile virus was compared in these stripped meninges with growth of virus in meninges still attached to skull. The results are shown in Table Ib and show that multiplication was similar in both systems. In view of this it was considered that cultures of meninges still attached to the skull would give similar results to meninges cultured alone, and in further experiments the latter system was adopted for ease of manipulation.

The basic experiment with West Nile virus was repeated 18 times (Tables I and II) and multiplication occurred on all occasions. Single growth curves were obtained with yellow fever virus and Semliki Forest virus.

Study 2. Six experiments were performed to see whether West Nile virus grew less well in meninges from immunized mice than in meninges from normal mice. The results are shown in Table II. Except for 2 pairs of titers (day 6 of Expts. 2 and 4), growth in normal meninges was at least equal to or greater than that in immune meninges. Also, the peak titers of the growth curves were higher and, in 4 of 6 experiments, by about 10-fold.

Table II (Expt. 1) shows virus growth in culture dishes containing both normal and immune meninges and compares this with growth in normal and immune meninges removed from the same animals but cultured separately. The "combined" growth curve lies half way between the curves obtained from cultures of normal and immune meninges alone.

In 1 experiment (Expt. 5 of Table II) normal or immune lymphocytes (5) were added to inoculated and uninoculated cultures after washing. The results show that virus grew less well in the presence of immune lymphocytes, whether the meninges were from an immune animal or not. Other experiments showed that the virus did not multiply in normal or immune lymphocytes

TABLE II. Comparison of West Nile Virus Multiplication in Normal and Immune Meninges in Culture.[a]

Expt.		Initial harvest after washing (zero time) (pfu/ml)	Days after inoculation (pfu/ml × 10^3)					
			1	2	3	4	6	8
1	Normal meninges	0.08	1	—	300	1300	2200	400
	Immune meninges	0.3	0.5	—	2.5	50	600	300
	"Combined" normal/ immune cultures	0.1	3	—	80	600	1200	30
2	Normal meninges	0.05	60	—	50	50	200	120
	Immune meninges	0.6	0.1	—	30	30	300	40
3	Normal meninges	0.07	—	0.6	30	4400	2400	0.16
	Immune meninges	0	—	0.06	24	3600	1600	0.04
4	Normal meninges	3.7	—	12	—	2500	140	—
	Immune meninges	0.125	—	12	—	200	200	—
5a	Normal (+ normal lymphocytes)	5	—	7.5	—	2500	630	—
	Immune (+ normal lymphocytes)	2.5	—	1.5	—	140	380	—
5b	Normal (+ immune lymphocytes)	0.2	—	150	—	550	8	—
	Immune (+ immune lymphocytes)	1.2	—	5	—	75	0	—

[a] Inoculum, 1 × 10^4 pfu/ml.

when cultured alone.

Study 3. Meninges from immune mice were cultured, both alone and together with pig kidney cells, for 6 days and medium was harvested daily and tested for virus infectivity. No virus was detected in the harvests from either simple culture or co-cultivation experiments.

Discussion. Organ cultures have been extensively used to study viral infections of the respiratory tract and skin (6), and more recently of the alimentary tract (2). There are no previous reports on virus replication in organ cultures of meningeal tissue.

This report shows that murine meninges in organ culture support the replication of 3 arboviruses. This organ culture system is easy to manipulate and permits an *in vitro* analysis of virus replication under conditions which are more like those *in vivo* than the standard monolayer tissue culture.

One important objective of these experiments was to investigate the nature of immunity to arbovirus infections. In these experiments the meningeal tissues were cultured for 24 hr prior to virus inoculation and the nutrient medium was changed either daily or on alternate days. Although some circulating antibody may have remained adherent to the meningeal cells, it seems more likely that in view of the initial washing procedure and frequent changes of medium, any circulating antibody would have been washed away by the time of maximum virus multiplication, *i.e.*, 3–5 days following infection. The results indicate that, despite the absence of circulating antibody, prior immunization conferred some degree of resistance on meningeal tissue.

The slight reduction in the yield of virus when immune and normal meninges were combined may have been due to the production of antibody by the immune meninges. Also the effect of the immune lymphocytes could have been due either to antibody secretion or to a direct attack on the virus particle. Clearly, more investigations are required before this can be determined.

No virus was recovered from meninges in these West Nile virus experiments and it is not, therefore, possible to relate the relative immunity in the meninges taken from immunized mice to the presence of virus antigen in the cells, although in yellow fever infections of mice virus can persist for long periods (7). The results indicate that immunity depends, to some degree, upon an acquired property of the meningeal cells, possibly resulting from the production of interferon triggered by an antigenic stimulus (8).

Summary. Replication of three arboviruses, West Nile, Yellow fever and Semliki Forest, occurs in organ cultures of mouse meninges. Replication of West Nile virus is reduced by prior vaccination of the animals. The addition of immune lymphocytes also reduces the yield of West Nile virus from the cultures.

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