Cross-Protection Between Venezuelan Equine Encephalomyelitis and Eastern Equine Encephalomyelitis Virus. (26702)

HENRY J. HEARN, JR. (Introduced by Arthur Brown)
U. S. Army Chemical Corps, Fort Detrick, Frederick, Md.

The viruses of the equine encephalitides have been reported to be related antigenically(1). Evidence of cross-protection among these viruses following immunization with inactivated viruses is lacking(2,3,4). However, immunity induced by injection of live virus appears to be cross-protective among certain of the group A arthropod-borne viruses. Stamm and Kissling(5) reported cross-protection between live eastern and western strains of equine encephalomyelitis virus in sparrows and horses, and Parks and Price (6) showed that guinea pigs injected with the live western strain resisted peripheral challenges with Sindbis virus and vice-versa. Casals(1) reported that 2 injections of live Chikungunya virus resulted in an appreciable resistance in mice to challenge by Semliki Forest virus. More recently it was found (7) that one injection of an attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus, strain 9t, enabled mice and monkeys to resist large lethal doses of the virulent homologous strain given intracerebrally, and that the high immunizing efficiency of the vaccine strain was related to a limited viral multiplication which was found in the absence of clinical illness(8). These findings suggested that cross-protection among group A arthropod-borne viruses should be examined further. In these experiments, mice immunized by either the subcutaneous, intraperitoneal, or respiratory route with 9t were challenged with serial dilutions of a chick embryo seed preparation of eastern equine encephalomyelitis (EEE) virus. In addition, experiments were carried out in which animals that were injected with EEE virus were challenged with a virulent VEE virus strain.

Materials and methods. Virus strains. The attenuated VEE virus, 9t, used to immunize mice was a tissue culture seed prepared after its isolation from a chronically infected L-cell culture followed by 9 passages in fresh L cells. This strain represents an additional passage in L cells of the CILV-8 strain which was described elsewhere(7). The virulent parent egg seed, PES, of VEE virus used to challenge immunized animals also has been described(7).

The EEE virus seed, EE-2, was isolated from the brain of a human case, passed 8 times in mouse brain and 3 times in chick embryos. In preparation of the 4th passage egg embryo seed, 0.1 ml of a 10^{-3} dilution of EE-2 was inoculated into the allantoic cavity of 10-day eggs. Embryos harvested at 24 hours were prepared as virus seeds in the same manner as for the PES strain(7).

Strain differences as determined by animal response to injection of virus. The differences in virulence of PES, 9t and EE-2 viruses were determined as follows: serial 10-fold dilutions of each strain were injected into 5- to 6-kg rabbits intraperitoneally (i.p.), into 200-to 300-g guinea pigs i.p. and into 4-day chicks subcutaneously (s.c.). Animals that withstood doses of 10^6 $MICLD_{50}$ of virus or less were considered as resistant to that virus; animals which succumbed to 10^2 $MICLD_{50}$ were considered susceptible.

Strain differences as determined by serum neutralization tests. Neutralization tests with anti-9t or anti-PES monkey sera or anti-EE-2 rabbit sera were used to verify differences among virus strains. They were performed in mice according to the method described elsewhere(7). Neutralization tests were also caried out in L-cell(9) cultures that had been grown for 48 hours in Leighton tubes containing medium 199(10) supplemented with 20% horse serum. Fifteen-hundredths (0.15) ml of serial 10-fold dilutions of virus in Bacto heart infusion broth (BHIB) were incubated for 30 minutes at room temperature with 0.15 ml antiserum which was diluted 1:1 in BHIB. The mixture was then added in 0.2 ml amounts to duplicate culture tubes containing 1 ml of

TABLE I. Comparative Resistance of Mice Challenged with a Virulent Strain of VEE Virus (PES) or EEE Virus (EE-2) after Immunization with Attenuated VEE Virus (9t).

Route of immunization	Challenge virus	Route of challenge	Titer $MICLD_{50}$ (log_{10}) of challenge virus in 9t immunized mice	Titer $MICLD_{50}$ (log_{10}) of challenge virus in Nonimmunized mice	Protective index* (log 10)
Intraperitoneal†	PES	Intraperitoneal	<1.6	8.9	>7.3
		Intracerebral	2.8	9.2	6.4
Respiratory‡		Intraperitoneal	<1.6	8.6	>7.0
Intraperitoneal	EE-2	Intraperitoneal	2.2	6.4	4.2
		Intracerebral	7.1	8.7	1.6
Respiratory		Intraperitoneal	3.5	6.6	3.1

* Difference in No. of LD_{50} resisted by immunized and nonimmunized animals.

† Immunization dose was $10^{3.4}$ $MICLD_{50}$. Similar results varying no more than 0.3 log in last column were obtained after subcut. immunization with 9t.

‡ Calculated immunization dose of $10^{3.4}$ $MICLD_{50}$ given by 5-min. exposure to aerosols of 9t.

tissue culture medium. Control tubes were inoculated with mixtures of virus and normal serum, or virus and BHIB.

The tubes were examined microscopically every 24 hours for 6 days for evidence of a cytopathic effect (cpe) due to virus multiplication in the cell monolayer. Virus titers were determined on the basis of the highest virus dilution which produced a cpe in 50% of the tubes and were expressed as number of $CPED_{50}$(11) per ml of virus suspension. The neutralization index was computed as the difference between $CPED_{50}$ titers obtained with normal serum or BHIB and those with antiserum. A neutralization index of less than 10 was considered not significant.

Tests for formation of plaques were carried out according to a method described elsewhere(12) except that neutral red was added in combination with a second agar overlay.

Virus immunizations. Twelve- to fourteen-gram mice were vaccinated with $10^{3.4}$ $MICLD_{50}$ of 9t given i.p. or s.c. Vaccinations by the respiratory route were carried out by exposing animals to aerosols of 9t for 5 minutes in a modified Henderson apparatus (13). Mice received maximum aerosol doses of $10^{3.4}$ $MICLD_{50}$, which were calculated on the basis of 100% retention of total number of $MICLD_{50}$ in the volume of air inhaled within a 5-minute exposure period. Rabbits and guinea pigs were immunized against EEE virus by an i.p. injection of 10^{8} and 10^{6} $MICLD_{50}$, respectively of EE-2; the hosts were resistant to these doses.

Virus challenges. Challenge doses of virus were administered to immunized animals at 21 days postvaccination. Controls consisted of animals which came from the same group as those that were immunized. They were, therefore, comparable to unimmunized animals with respect to age, weight and length of time in contact with the same laboratory cage facilities. Mice immunized i.p., s.c., or by aerosol exposure were challenged i.p. or i.c., with 10-fold dilutions of EE-2 or PES diluted in BHIB. Challenges with $10^{5.3}$ $MICLD_{50}$ of PES were administered i.p. in rabbits and guinea pigs which had received an injection of EE-2.

Results. The results presented in Table I indicate that the attenuated VEE strain effectively protected mice not only against a large challenge dose of virulent VEE virus but also against a substantial dose of virulent EEE virus. The protective responses were elicited after immunization with the attenuated strain administered by the i.p., s.c., or respiratory route. As expected, the protective effect was greater when the immunized animals were challenged with the virulent homologous strain than when challenged with the heterologous EE-2 virus.

As shown in Table II, rabbits and guinea pigs, which were naturally refractory to EE-2, resisted a challenge dose of $10^{5.3}$ $MICLD_{50}$ of the virulent PES. Control animals which had not previously received an injection of EE-2 died 3 days after a PES challenge of 10^{2} $MICLD_{50}$.

Before these data were finally assessed,

TABLE II. Protection of Rabbits and Guinea Pigs against VEE Virus after Injection of Live EEE Virus.

Challenge dose of VEE virus ($MICLD_{50}$)*	No. dead/Total No. challenged with VEE virus			
	Immunized with EEE virus		Unimmunized controls	
	Rabbits†	Guinea pigs‡	Rabbits	Guinea pigs
$10^{2.0}$			2/2	8/8
$10^{5.3}$	0/2	0/10		

* VEE virus challenge by intraper. route.
† 10^8 $MICLD_{50}$ of EEE virus given intraper.
‡ 10^6 $MICLD_{50}$ " " " " " .

however, tests were carried out to demonstrate that the virulent PES, the attenuated 9t strain which was derived from it, and EE-2 virus preparations represented separate and distinct etiologic agents. Such tests were performed to eliminate the possibility that contamination of virus strains might have been responsible for the cross-protection which was observed. In Table III, a number of distinguishing properties of each virus are listed. PES virus was lethal for mice, rabbits and guinea pigs when doses of 10^2 $MICLD_{50}$ were given i.p. but nonlethal for 4-day chicks when doses of 10^8 $MICLD_{50}$ were given s.c. The attenuated strain was nonlethal for mice, rabbits, guinea pigs and 4-day chicks when doses of 10^6 $MICLD_{50}$ were given i.p. With EE-2 virus, rabbits resisted 10^8 $MICLD_{50}$ given i.p. Guinea pigs resisted 10^6 $MICLD_{50}$ by the i.p. route but doses of 10^7 $MICLD_{50}$ or greater produced illness and death. A dose of 10^2 $MICLD_{50}$ of EE-2 given s.c. was lethal for 4-day chicks. The 3 strains were also distinguishable by plaque tests with chick fibroblasts. The size of plaques produced by PES was 3-7 mm, by EE-2 it was 2-3 mm and by 9t, 0.5-1.5 mm. In addition, immune monkey sera prepared against 9t or PES neutralized both strains in tissue culture and mice but failed to neutralize EEE virus. Conversely, immune rabbit sera prepared against EE-2 virus neutralized this strain in mice but failed to neutralize 9t in tissue culture.

Discussion. The results of these experiments indicated that immunization of mice with the attenuated 9t strain of VEE virus protected them against a large lethal dose of the homologous virulent strain and against substantial doses of the heterologous EEE virus. Similarly, cross-protection against a lethal VEE-virus challenge was evident in rabbits and guinea pigs immunized with living EEE virus. It was necessary to test for cross-protection between VEE and EEE virus strains in at least 2 host-species because of the inherent susceptibility of mice to EE-2 and because rabbits and guinea pigs were naturally resistant to challenge with EE-2. In the mice, immunization was not readily accomplished with the live antigen while in the latter hosts it was difficult to assess an acquired resistance in animals that were inherently refractory to the challenge agent.

It is of particular importance to note that antisera from laboratory animals infected with EEE or VEE virus did not cross-react in serum-neutralization tests that were carried out in mice or in tissue cultures. In the latter system, neither an inhibition of a cpe (cell lysis) in fluid cultures nor an inhibition of formation of plaques was observed. Direct experimental evidence that explains the

TABLE III. Properties Which Distinguish Parent Egg Seed (PES) and Attenuated (9t) Strains of VEE Virus and EEE (EE-2) Virus as Distinct Etiologic Agents.

Property	Virus strain		
	PES	9t	EEE
Lethality* for mice i.p.	+	—	+
" " guinea pigs i.p.	+	—	—
" " 4-day chicks s.c.	—	—	+
" " rabbits i.p.	+	—	—
Neutralized by anti-EEE sera	—	—	+
" " anti-9t or anti-PES sera	+	+	—
Plaque size on chick fibroblasts	Large	Minute	Intermediate

* Lethality (+): doses of 10^2 $MICLD_{50}$ were lethal by route indicated.
Lethality (—): doses of 10^6 $MICLD_{50}$ were nonlethal by route indicated.

mechanism of the cross-protection in animals. therefore, is lacking.

Summary. Mice were capable of withstanding a large challenge dose of virulent Venezuelan equine encephalomyelitis virus and a substantial challenge dose of eastern equine encephalomyelitis virus after immunization with an attenuated 9t strain of VEE virus. This resistance was elicited after administration of the 9t strain by the intraperitoneal, subcutaneous, and respiratory routes, the latter being accomplished by short exposures to aerosols of the attenuated strain. Rabbits and guinea pigs, which were refractory to infections with EEE virus, were protected against lethal doses of the heterologous virulent VEE virus.

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Thermal Degradation of Foot-and-Mouth Disease Virus Into Infectious Ribonucleic Acid.* (26703)

HOWARD L. BACHRACH
Plum Island Animal Disease Laboratory, Agricultural Research Service, Animal Disease and Parasite Research Division, U. S. Dept. of Agriculture, Greenport, N. Y.

Infectious ribonucleic acid (RNA) was first obtained from foot-and-mouth disease virus (FMDV) by phenol extraction(1,2). Mussgay(3) presented evidence that FMDV dissociates upon acidification at pH 5.0 into RNA and protein subunits. Bachrach(4) reported that FMDV, which appeared to be completely inactivated by boiling at 100°C for 5 minutes, still yielded infectious RNA upon extraction with phenol; phenol-derived RNA was not completely inactivated after heating at 85°C for 5 minutes; and (5) FMDV-RNA has a wider pH stability spectrum than FMDV preparations that contain ribonuclease (RNase).

The research reported here shows that heating degrades FMDV into infectious RNA whenever environmental RNase is simultaneously inactivated with hot sodium dodecylsulfate (SDS) or when Rnase has been previously removed.

Materials and methods. FMDV, type A, strain 119, which had been passaged in calf-kidney cultures 100 times, was used. Virus was concentrated 100-fold from tissue culture fluid containing 2% bovine serum by precipitation with 20% methanol at 0°C, resuspension in 1:1 Tris-Hanks' solution containing 0.5% bovine lactalbumin hydrolyzate (BLH), dialysis to remove methanol, and clarification at 15,000 rpm for 15 minutes. Such concentrates still contained RNase(6). Virus from which RNase was to be quantitatively removed was propa-

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