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An Attenuated Variant of Eastern Encephalitis Virus: Biological Properties and Protection Induced in Mice

By

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Summary

Wild type Eastern equine encephalitis virus (E) was compared with a mutant (E_m) derived from it. The latter was tested as an attenuated vaccine in mice. They differed in the following properties: E_m formed smaller plaques on chick embryo (CE) cell monolayers and, unlike E, did not plaque on mouse embryo (ME) monolayers. Further, E_m had a longer latent period and attained a lower peak titer than E after infection of CE cells, was more sensitive than E to chick interferon, and was less virulent for mice (SC and IP routes) and hamsters (IP route) than E. Both viruses were similar in several other properties tested.

The mutant was found to induce a gradient in the specificity of protection in mice against challenge by selected viruses after a single subcutaneous injection of living virus. The protection was best against autologous (E_m) challenge, was next best against challenge by the virulent parent (E) virus, but was not demonstrable against cross challenge by Venezuelan encephalitis (V) virus. Conventional hemagglutination-inhibiting (HI), complement-fixing (CF), and neutralizing (N) antibodies could not be detected in E_m-immunized mice even when fresh monkey or guinea pig serum was included in N tests to provide complement and/or accessory factor(s). However, N antibodies were detected in protected mice by an indirect antiglobulin test. Passive protection by serum or ascites fluids (a.f.) was characterized by a lower but otherwise similar protection gradient like that found after active immunization with virus as described above. Interferon was not detected in the a.f. used for passive protection, nor was heterologous interference evident in E_m immunized mice challenged 18 days later with vaccinia or vesicular stomatitis virus. Immunized mice that survived autologous (E_m) challenge showed broadened protection against a second chal-

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lenge by parent E virus, and cross protection against V virus. This typical protection was associated with the presence of HI and conventional N antibodies, except for V which showed no detectable neutralizing antibodies by either a standard or antiglobulin technique.

1. Introduction

There has been a continuing interest for several decades in the genetics of viruses, including the induction and/or selection of attenuated variants as potential live vaccines. Such viruses have been selected after extensive animal or cell culture passages (*e.g.* 1, 16, 29), from small plaque mutants that arise either spontaneously or after induction by chemical mutagens (*e.g.* 10, 31), and from temperature sensitive mutants that are isolated directly after mutagenesis and/or after many passages in cell cultures that were incubated at temperatures below 37° C (*e.g.* 5, 14, 20).

In the course of some genetic studies in our laboratory, a minute plaque mutant (E_m) of Eastern encephalities (E) virus was obtained by Dr. A. Richter by direct plating on chick embryo (CE) cells after nitrous acid treatment. One of our immediate objectives was to characterize the mutant, including testing in higher animals. It was found that the mutant differed from the parent in a number of properties. Further, it induced a gradient in specificity of protection in mice against challenge by selected viruses after a single immunizing dose of living virus, and induced broadened protection after two immunizations.

2. Materials and Methods

2.1. Viruses, Methods of Assay, and Cell Culture

The parent strain of Eastern encephalitis virus (E) and the Trinidad strain of Venezuelan encephalitis (V) virus used in this work, and its methods of assay by the monolayer plaque technique in chick embryo (CE) cells, and in animals have been described previously (3). The small plaque mutant (E_m) was kindly supplied to us by Dr. A. Richter who isolated it after treatment of E with nitrous acid to a survival of 0.01 per cent. Chick embryo (CE) cell culture stocks of the E_m, E, and V were made from supernatants containing virus that had been plaque-purified three times and inoculated into chick embryo (CE) cell cultures at a dose between 1—10 p.f.u. per plate. In a few challenge experiments, a mouse neutrotropic strain (IHD) of Vaccinia (Vac) virus, obtained from Dr. F. Schabel and the Indiana strain of vesicular stomatitis (VS) virus, obtained from Dr. R. R. Wagner, were used. The preparation and use of monolayers of CE cells have been described previously (3). The same procedure was used to prepare and maintain mouse embryo (ME) cells from embryos at the 18th day of gestation.

2.2. Chick Interferon Preparation and Assay

The methods for producing interferon (IF) in chick embryos with influenza virus and assaying the IF by 50 per cent plaque reduction with VS virus in CE cells have been described (32). The comparative sensitivity of E and E_m to IF was determined on the same day. The criteria used to determine that the inhibitor was indeed chick IF were: 1. resistance to 56° C, 1 hour and to pH 2.0; 2. susceptibility to trypsin; 3. failure to inactivate E virus *in vitro* (in the absence of cells); 4. the abolition of interference of VS virus multiplication in cultures of CE cells by actinomycin D; 5. the inability to interfere with VS virus multiplication in heterologous mouse L cells.

2.3. Mouse Interferon

Untreated normal and "immune" ascites fluids (4) were tested for interferon (IF) activity in mouse L cells by the criterion of 50 per cent reduction in VS plaques, starting with undiluted fluids (32). However, observations were also made in the same experiment for a reduction in plaque *size* when no IF activity was demonstrable, since a reduction in plaque size is frequently observed in doubling dilutions beyond the 50 per cent end point that is otherwise based on a reduction in plaque numbers (32).

2.4. Heat Inactivation and Maximum Temperature of Plaque Formation in CE Cells

The heat inactivation of partially purified virus was carried out at 50° C (21). The maximum temperature for plaque formation was determined using a temperature gradient plate (3).

2.5. Antibodies

The quantitative determination of antibodies was made on serum and/or cell free ascites fluids (a.f.). Neutralizing antibodies were determined by a standard (N) and a more sensitive indirect antiglobulin neutralization (AGN) technique in which 10^3 p.f.u./ml of virus was used in the test (4). Antiglobulin will neutralize "incomplete" (*e.g.* papain digested) antibody-virus complexes, or other infectious virus-antibody complexes [see literature review in (3)]. The goat antimouse globulin used in our tests had a precipitin titer of 1:64. Normal a.f. or serum as controls in both the N and AGN tests were obtained from sham (diluent) inoculated mice of the same group of animals used in the experiments to obtain a.f. or serum from virus-immunized and/or challenged mice. Additional controls were frequently included in AGN tests, but are not shown in the tables because of brevity, and because they were consistent in at least three separate experiments months apart using different experimental conditions. The additional controls consisted of the following: Virus + BHIB[1] + amγg[2]; Virus + "immune" a.f. or serum[3] + BHIB; Virus + "immune" a.f. or serum + goat anti-human γ-globulin; Virus + BHIB + BHIB. On occasion a N test was carried out in mice by both constant virus-varying serum dilution and constant serum-varying virus dilution (13). Complement-fixing, and hemagglutination-inhibiting antibodies were assayed by standard techniques (13).

2.6. Active Immunization and Challenge

Single immunizations with E_m were performed by injecting approximately 5×10^3 plaque forming units (p.f.u.) into 8—12 gm weanling Swiss albino mice by the subcutaneous (SC) route. It has been determined in our laboratory that 1 p.f.u. of E_m, E, or V viruses on CE cells is approximately equal to 1 mouse median lethal dose by the intracerebral route [1 MIC LD_{50} (3)]; the median lethal dose was determined by the method of Reed and Muench (25). Mice were observed for 10 days. Only deaths occurring after 24 hours were considered virus-specific. Even with very high doses, however, the mice do not usually begin to die until about 48 hours post injection.

Challenges of mice immunized once were carried out by either the IC route or the IP route. The survivors in certain challenge experiments (*e.g.* E_m immunized by the SC route and challenged with E by the IP route) were considered to be equivalent to doubly immunized animals in experiments in which a second challenge (third injection) was given later. Protection in active immunization experiments was expressed as a protective index (PI). This was calculated as the $Log_{10}LD_{50}$ titer of the control-$Log_{10}LD_{50}$ titer of the test. We, like others, have found in our laboratory that a PI of 0.7 to be marginally significant, but 1.0 or greater to be significant. Ten mice per decimal dilution were used to calculate LD_{50}.

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[1] BHIB Difco beef heart infusion broth.

[2] Goat anti-mouse gamma globulin.

[3] From known immune mice with good N titers.

2.7. Passive Immunization, Challenge, and Protection

Passive immunization was carried out by injecting 0.4 ml of "immune" and normal a.f. into mice by the IP route and, in one experiment, 0.1 ml by the intravenous route. The "immune" fluids were obtained from mice that were immunized about 17 days previously with E_m by the SC route from the same group of mice in which a protection gradient (i.e., differences in response to challenge with E_m, E, and V) was observed in a repeat experiment. The mice were challenged 1 day after a.f. injection by both the IC and IP routes with a dose of 500 LD_{50} of E_m, E, or V viruses. It is well known that the LD_{50} of a virus stock of certain arboviruses (*e.g.* E), is dependent on the route of inoculation, on the age of the mice (2, 22) and other subtle factors; the peripheral routes (IP, SC) are least sensitive for E, and the IC route the most sensitive.

In a preliminary experiment, it was found that protection after one immunization as expressed by a PI was marginal to low. Therefore, another method using two criteria was chosen to test for an anticipated low level of protection. These were: 1. differences in the percent protection of "immune" and control animals that were challenged with an appropriate single dose of virus and, 2. differences in the average day of death that was calculated on the basis of observations made twice a day, 10—14 hours apart. The mice were observed for 14 days in passive protection experiments. Twenty to twenty-five animals per group were employed in all experiments of this kind that made use of a single dose of virus.

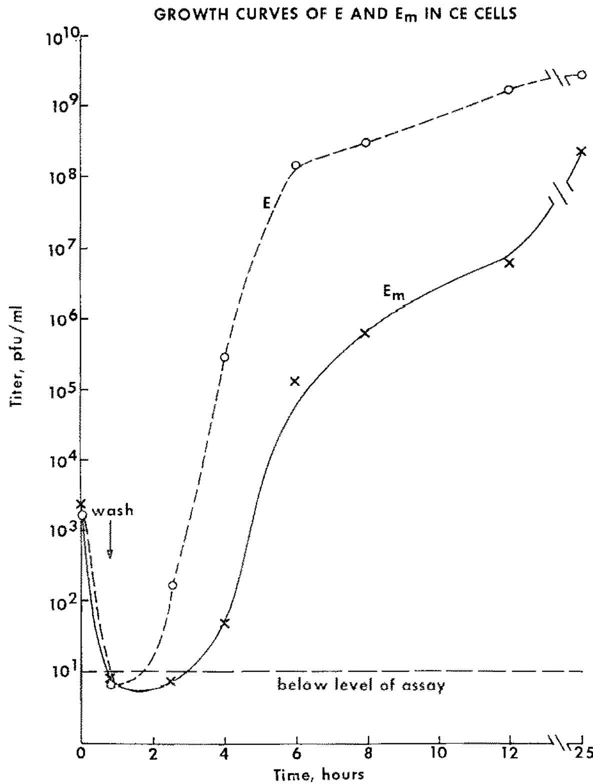

Fig. 1. CE cultures were infected at an input multiplicity of 1 p.f.u./cell incubated at 4° C for 30 minutes, washed three times with Saline A, re-incubated at 37° C for 45 minutes to remove eluted virus, washed three times with Saline A and then incubated at 37° C. The 0 hour sample was taken before the incubation for 45 minutes

3. Results

3.1. Growth Curves

A number of experiments were carried out to characterize the mutant, and to compare it to its parent. The growth curves of both E and E_m were determined by infecting monolayers of CE cells in a 60 mm dish with input multiplicities of 1 and 10. After 30 minutes adsorption at 4° C, the cultures were washed three times with Saline A to remove unadsorbed virus and fresh medium added which contained 10 per cent calf serum, lactalbumin hydrolysate, and yeast extract (3). After 45 minutes incubation at 37° C, the cultures were washed twice with Saline A to remove eluted virus, and re-fed. Pooled samples were taken from three plates at various time intervals, then frozen and stored at −65° C, and assayed within a few days. The results obtained at both multiplicities were similar in character and are shown for the lower multiplicity in Figure 1. They indicate

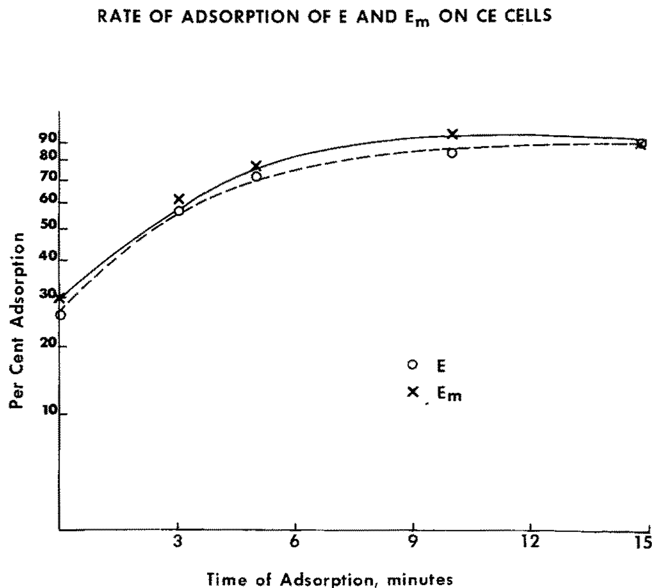

Fig. 2. CE cultures were infected at an input multiplicity of 1 p.f.u./cell at 4° C. Sequential supernatant samples for p.f.u. assay were taken within 30 seconds, and at intervals thereafter

that compared to E, E_m has a longer latent period, but a shorter rise period, and attained a lower peak titer. No attempt was made to "dissect" the latent period of each virus beyond adsorption, *i.e.*, penetration, eclipse, uncoating, etc., in order to define the precise areas of difference between them. Figure 2 shows that there were no significant differences in the rates of adsorption at 4° C as determined by p.f.u. titers remaining in the supernatant after infection at an input multiplicity of one p.f.u. per cell.

Actinomycin D (1 µg/ml) added one-half hour previous to infection had no perceptible effect on the growth curves of either virus, thus helping to rule out that differences in the growth curves were due to differences in interferon (IF) induction and/or sensitivity to IF action (32).

Interference experiments carried out with E and E_m showed that E_m could not interfere with E or vice versa unless the interfering viruses were given a time advantage of 3 hours or greater, or a high multiplicity advantage (33). Since the virus stocks were made after three plaque purifications and very low inocula into CE cells, the presence of defective interfering particles in E_m stocks was considered unlikely (18, 27, 28). The differences in the growth curves of the two viruses remain unexplained but may be related to specific differences in their intracellular replication beyond adsorption which we did not investigate further.

3.2. Plaques and IF Sensitivity

The mutant was selected originally on the basis of plaque size on CE cells. This was studied in more detail by comparing the plaque size on CE cells and on ME cells of E_m and E under agar overlays. Plaque diameter ranges are given for 90 per cent of the plaques measured, since only about 10 per cent of the plaque diameters fell outside the ranges given. The results in Table 1 show that E plaques are approximately 3 times the diameter of E_m plaques on CE cells after 48 hours of incubation. After 72 hours of incubation, E_m continues to increase only very slightly compared to E. E_m did not form plaques at all on ME cells even after 3 days of incubation (Table 1). By contrast, E formed plaques of 1.0–2.0 mm in diameter in 48 hours on ME cells but at 10 per cent of the efficiency of CE cells. Table 1 also shows that E_m is three to six times more sensitive than E to chick IF. This was confirmed in repeat experiments on the same preparations.

Table 1. *Comparative Plaquing Properties of E and E_m*

Virus	Plaque Diameters[a] (mm) on		IF titer[b]	
	CE cells	ME cells	Prep A	Prep B
E	2.5—3.5	1.0—2.0	1:1280	1:640
E_m	0.5—1.0	No plaques (48 or 72 hours)	1:5120	1:10, 240

[a] After 48 hours incubation.
[b] Nearest doubling dilution that reduced the number of plaques to 50 per cent of that obtained with control (normal) chick allantoic fluid; doubling dilutions were made beginning with a 1/10 dilution.

3.3. Heat Inactivation, t_f-max, and Cross Neutralization

A number of additional properties of E and E_m were compared. These included the following: rate of inactivation of virus at 50° C; the t_f-max; cross neutralization. The results showed no significant differences between E_m and E. In the cross neutralization tests, a number of antisera against living and killed E_m and E from mice and rabbits were used in the test.

3.4. Comparison of Virulence (LD_{50} titers) in Animals[1]

The next experiment compared the virulence of the two viruses by titrating a CE cell culture stock of each in 8—12 gram weanling mice by three routes of inoculation (IC, IP, SC), and in embryonated eggs by three routes of inoculation (YS, All, Am), and in hamsters by the IP route only. The results of this experiment are shown in Table 2. Even taking into account the lower p.f.u. titer of E_m in CE cells, it may be seen that E_m is less virulent than E for mice by the SC route and the IP route, and for hamsters by the IP route. E_m does not appear to differ significantly from E in virulence for mice by the IC route, or for embryonated eggs by any of the three routes chosen.

Table 2. *Comparative LD_{50} Titers of E and E_m in Animals*

Virus	CE cell stock (Log_{10} p.f.u./ml) on CE cells	Mice[a] IC	Mice[a] IP	Mice[a] SC	Hamsters[a] IP	Emb. Eggs[a] YS.	Emb. Eggs[a] All.	Emb. Eggs[a] Am.
E	9.3	9.4	6.6	6.2	9.2	8.4	6.4	9.0
E_m	8.5	8.7	2.0[b]	<1.0	5.2	7.0	5.6	8.3

[a] LD_{50}/ml ($\log_{10}$).
[b] Erratic results frequently obtained for E_m by IP route only, even with different stocks.

3.5. Single Immunizations with E_m

Since E_m was partially attenuated for mice, the next series of experiments were performed to test whether living virus could be used as a vaccine for protection against parent E virus, and for cross protection against V virus. Mice (8—12 gm) were each immunized with 5×10^4 plaque forming units (p.f.u.) by the subcutaneous (SC) route. Enough mice were immunized so that several ten-fold dilutions of challenge virus could be given 10 mice per dilution, for each route of challenge. Together with sham (diluent) "immunized" controls, a PI could be calculated. Additional mice in the same experiment were sham- and virus-immunized so that samples of a.f. and serum could be taken for serological assays. Challenges were carried out at 14 and 21 days after immunization with E_m, E, and V by the IC and IP routes. The results were essentially similar for each period of challenge except that protection was slightly greater at 21 days. Table 3 shows the results of challenge at 14 days. E_m immunized mice were strongly protected against autologous (E_m) challenge by the sensitive IC route, a route

[1] If a quantitative estimate of "virulence" for comparison of E_m with E is desired, it can be obtained by calculating a ratio of any LD_{50} titer in any animal to the p.f.u. titer. The precision of such estimates is limited by the LD_{50} titers, as opposed to the p.f.u. titer, because of the well known greater variability of the former. In our hands, LD_{50} titers using mice, eggs and hamsters are usually good to $\pm 0.3 \log_{10}$ units (e.g. IC route in mice) depending on the animal and route of inoculation. The exception is E_m in mice by the IP route discussed above, which gives erratic results, such that, sometimes, LD_{50} titers cannot be meaningfully calculated.

where E_m is highly virulent in unimmunized mice. Protection against challenge by virulent E is evident only by the IP route and not the IC route of challenge. There is no evidence of protection against V by either route of challenge.

Table 3. *Gradient in Specificity of Protection among E_m, E and V after Immunization with E_m*

Immunized with (route)[a]	Challenged with (route)	LD_{50}/ml	Protective index[b, c]
Control 1	E_m (IC)	$10^{8.5}$	
Control 2	E (IC)	$10^{9.7}$	
Control 3	V (IC)	$10^{10.1}$	
Test 1 E_m (SC)	E_m (IC)	$10^{4.0}$	[4.5] [c]
Test 2 E_m (SC)	E (IC)	$10^{9.6}$	0.1
Test 3 E_m (SC)	V (IC)	$10^{10.0}$	0.1
Control 4	E_m (IP)	$10^{2.6}$[d]	
Control 5	E (IP)	$10^{6.8}$	
Control 6	V (IP)	$10^{9.3}$	
Test 4 E_m (SC)	E_m (IP)	$< 10^{1.0}$[d]	[1.6—2.6] [d]
Test 5 E_m (SC)	E (IP)	$10^{3.0}$	[3.8] [c]
Test 6 E_m (SC)	V (IP)	$10^{9.0}$	0.3

[a] 1 p.f.u. = 1 $MICLD_{50}$. Controls consisted of sham (diluent) injected mice.
[b] Protective index = $Log_{10}LD_{50}$ of control—$Log_{10}LD_{50}$ of test.
[c] Squares are placed around those figures which show significant protection.
[d] Variable and erratic (4).

3.6. Serological Tests

Ascites fluids and serum samples were obtained from E_m immunized mice at 14 and 21 days and assayed for HI, CF and plaque and mouse N antibodies beginning at 1/10 dilutions when constant virus-varying serum dilutions were used in plaque tests in CE cells and in mice by LD_{50}. In mice, the N test was also carried out by using undiluted serum and varying virus dilutions. No antibodies were detected by any of the methods even for N antibody with "accessory factors" added (12).

Since it has been shown that the AGN test is frequently more sensitive for the detection of (presumably) neutralizing or "incomplete" antibody (4), it was used with the same fluids which failed to detect antibodies by the standard methods described earlier. The results showed that only a.f. taken from E_m immunized mice provided evidence of neutralizing antibody when tested against E_m and E but not against V, as reported previously (4).

3.7. Passive Protection Tests

Since the mice immunized once with E_m showed significant PIs, even in the absence of neutralizing antibody detectable by standard techniques, it seemed of interest to see whether the a.f. from immune animals could confer protection against challenge in passive protection tests. The first experiment was carried

out in a direct imitation of the challenge protection experiment shown in Table 3 in which PIs were calculated. Challenges were carried out 24 hours after passive immunization with "immune" and normal a.f. The results showed a marginal PI for E_m challenge by IC route (0.9) and a low but significant PI for E challenge by the IP route (1.9). No other protection was evident. Therefore, the second method described in section 2.7. for demonstrating anticipated low levels of protection was used. In this experiment, the passive immunization was carried out exactly as in the previous experiment, but a single challenge dose was chosen. The criteria used to evaluate protection were the percent survival and the average day of death. The results in Table 4 show significant passive protection con-

Table 4. *Percent Passive Protection and Average Day of Death with Ascites Fluids from E_m Immunized and Control Mice*

Treatment[a]	Challenged with/route[c]	Percent survivors	Approximate A.D.D.[b]
E_m—"immune" a.f.	1000 MIC LD_{50} E_m/IC	[70][d]	[9.0]
	1000 MIC LD_{50} E/IC	0	4.0
	1000 MIC LD_{50} V/IC	0	3.5
	1000 MIP LD_{50} E/IP	[95]	[8.0]
	1000 MIP LD_{50} V/IP	0	4.0
Normal a.f.	1000 MIC LD_{50} E_m/IC	0	4.0
	1000 MIC LD_{50} E/IC	5	4.0
	1000 MIC LD_{50} V/IC	0	3.5
	1000 MIP LD_{50} E/IP	10	5.5
	1000 MIP LD_{50} V/IP	0	4.0
No treatment	1000 MIC LD_{50} E_m/IC	0	3.5
	1000 MIC LD_{50} E/IC	0	3.5
	1000 MIC LD_{50} V/IC	0	3.5
	1000 MIP LD_{50} E/IP	10	5.0
	1000 MIP LD_{50} V/IP	0	4.0

[a] Ascites fluids (a.f.) given by IP route 24 hours before challenge (20 mice per group).
[b] Average day of death on basis of observations twice daily, 10—14 hours apart.
[c] The LD_{50} units used for challenge for each virus by the routes indicated vary in their p.f.u. content depending on the virus and route.
[d] Squares are placed around those figures which show significant protection.

ferred by a.f. taken from E_m immunized mice against challenge by E_m virus (IC route), and E virus by the IP route only, but not against challenge by V virus by either route. Passive protection test results also demonstrated, therefore, a gradient in the specificity of protection that resulted after a single immunization with E_m.

3.8. Heterologous Interference and Interferon

Both the active immunization experiments and the passive protection experiments failed to show cross protection against V virus, a virus that is approximately 8—10 fold less sensitive than E to interferon (19, and our unpublished

results). For this and other reasons, it seemed of interest to test E_m immunized mice and a.f. taken from them for heterologous interference, presumably mediaated by IF. For this test, mice were immunized once with E_m and challenged 18 days later by the IC route with VS or Vac viruses, both of which are susceptible to IF in cell culture. Furthermore, it has been previously shown by a colleague (15), that mice immunized with an attenuated variant of V virus, induced heterologous interference to the IHD strain of Vaccinia virus injected by the IC route beginning at one but not after four days, a result typical of non-specific heterologous interference (7, 26). However, we had to consider that an attenuated variant might induce IF or interference against heterologous viruses (*e.g.* by a low grade chronic infection). The results presented in Table 5 show

Table 5. *Test for Non-Specific Cross Protection (Interference) in Immunized Mice*

	Challenge[a] virus	Route of challenge	P.I.[d]
Controls[b]	E_m	IC	3.8
	E	IP	2.9
Test[c]	Vaccinia (IHD)	IC	0.2
	VSV	IC	0.3
	E	IC	0.3

[a] Challenge—18 days after one SC injection of E_m.
[b] Positive specific protection controls.
[c] Viruses known to be susceptible to interferon.
[d] $\text{Log}_{10}\ LD_{50}$ titer in control mice—$\text{Log}_{10}\ LD_{50}$ titer in immunized mice.

that E_m immunized mice failed to show significant protection against IC challenge by VS and Vaccinia viruses but that they did show significant resistance in the positive control challenges by E_m (IC route) and E viruses (IP route only). These results suggested that heterologous interference mediated by IF was probably not involved in passive or active protection. Nevertheless, when activity was sought by tests in cell culture, the tests made from normal and "immune" a.f. taken from the E_m immunized mice (either 14 or 18 days after injection) in this and other experiments failed to show any evidence of IF sensitive heterologous viruses (VS and Vac). Furthermore, the protection demonstrable after active immunization could be reproduced (albeit at a lower level) in passive protection experiments, and could be associated with AGN antibody but not with antibody detectable by HI, CF, or standard N tests.

It has been amply demonstrated (6, 8, 15, 17) that some single and, especially, double immunizations among the Group A arboviruses broaden the protection against other members of the group. Some of the cross protection is even demonstrable among those that show no cross neutralization either in the serum samples taken at the time of challenge, or from high potency antisera made against each (living *or* killed) virus separately. Therefore, experiments were undertaken to test for increased homologous and cross protection among survivors of an experiment similar to the first experiment (Table 3). A PI was calculated on doubly immunized mice, in which specific doses of the designated virus was adminis-

tered before the test challenge. The challenge injection was given 7 days after the second immunization. The protocol and results of this experiment may be seen in Table 6.

The results indicate that double immunization results in a broadened and increased protection, including cross protection against V virus, which was not observed in mice immunized only once with E_m.

Table 6. *Broadened and Increased Level of Protection against E and V Virus after Double Immunization—by Protective Index*[a]

Description	1st immunization Virus-Route-Quantity	PI[a] (after challenge with Virus-Route)	2nd immunization Virus-Route-Quantity	PI[a] (after challenge with Virus-Route)
Test 1	E_m-SC 5000 p.f.u.	3.8 (E_m-IC)	E_m-IC 1000 p.f.u.	4.9 (E_m-IC)
Test 2	E_m-SC 5000 p.f.u.	0.2 (E-IC)	E_m-IC 1000 p.f.u.	4.1 (E-IC)
Test 3	E_m-SC 5000 p.f.u.	3.3 (E-IP)	E_m-IC 1000 p.f.u.	4.5 (E-IP)
Test 4	E_m-SC 5000 p.f.u.	—0.2 (V-IC)	E_m-IC 1000 p.f.u.	1.1 (V-IC)
Test 5	E_m-SC 5000 p.f.u.	0.2 (V-IP)	E_m-IC 1000 p.f.u.	2.6 (V-IP)

[a] PI Log_{10} LD_{50} titer of sham inoculated controls—Log_{10} LD_{50} titer of test. PI of 1.0 is considered significant.

3.9. Serological Assays in Doubly Immunized Mice

It was previously observed that conventional HI and N antibody could not be detected in mice that were immunized only once with E_m, although such mice showed a gradient in specificity of protection against subsequent challenge by E_m, E, and V viruses. In view of the broadened protection demonstrated in doubly immunized mice (Table 6), it was of interest to see whether conventional HI and N antibody could now be detected in the animals just prior to challenge. Serum samples and a.f. were taken from groups of mice that were: 1. Sham immunized-samples taken at 25 days; 2. immunized once with 5000 p.f.u. of E_m by the SC route followed 18 days later with 1000 p.f.u. of E_m by the IC route-samples taken at 25 days. The results in Table 7 show that only after double, but not single, immunization could HI and standard N antibody be detected. However, neither N or AGN antibody could be detected against V virus. The latter results are consistent with those of others who have found HI antibody but not cross-neutralizing antibody in experiments which nevertheless demonstrate cross protection among those Group A arboviruses that have not been shown to share antigens demonstrable in N tests (6, 8, 15, 17). The viruses used here are known to share hemagglutinating antigens but these are believed to play little, if any, role in inducing protection. Finally, the same fluids that were tested for cross neutralizing antibody were tested for IF activity as was done previously for singly immunized mice. Again, no IF was detected.

Table 7. *Level of Antibody in Mice Singly and Doubly Immunized with E_m*

		Antibody test and virus			
		HI[a]		N[b]	
Immunizations and route	Fluid used	E	V[c]	E	E_m
None—Sham	Serum[d]	<10	<10	<10	<10
None—Sham	a.f.[d]	<10	<10	<10	<10
One—SC+Sham	Serum	<10	<10	<10	<10
One—SC+Sham	a.f.	<10	<10	<10	<10
Two—SC+IC (18 days)	Serum	40	<10	160	160
Two—SC+IC (18 days)	a.f.	40	<10	160	320

[a] Reciprocal of dilution that prevents hemagglutination by a mouse brain E antigen (12).

[b] Reciprocal of nearest doubling dilution that reduces plaques to 50 per cent of controls in a standard N test.

[c] Because no standard N antibody was demonstrated against V virus, the more sensitive AGN test (4) was also used. No AGN antibody was detected.

[d] Fluids tested were taken at 18 days for sham immunized and singly immunized mice, and at 25 days for doubly immunized mice.

4. Discussion

A comparison of a number of biological properties between the mutant E_m[1] and its wild type parent revealed many similarities but some striking differences. With respect to the latter, E_m was found to form smaller plaques than E on CE monolayers; further, in contrast to E, E_m did not plaque on ME monolayers. E_m showed differences from E in its 'growth curve', was more sensitive than E to chick interferon, and was significantly less virulent for mice and hamsters by certain routes of injection. It is unlikely that the differences represent a pleiotropic effect of a single mutation, since some of the properties almost certainly involve different functions of the virus. For example, interferon sensitivity which involves an inhibition of an early event in virus nucleic acid replication (12), or growth curve differences, are unlikely to have any direct correlation or resemblance to sensitivity to an agar inhibitor which affects the virus protein coat (9, 10). The difference in several properties between E and E_m is likely, therefore, to be due to two or more mutations in the virus because of nitrous acid treatment and/or selection during plaque purification and preparation of stock.

E_m was found to be avirulent by the SC route and is apparently able to induce a gradient in specificity of protection against challenge after a single SC injection in mice. The gradient is expressed as follows: 1. substantial protection against death following autologous (E_m) challenge by the IC route, but not against the virulent parent (E) or the heterologous V by the same route; 2. protection against parent E by the less stringent IP route but not against heter-

[1] A small plaque variant of E used in immunization studies has been reported but has not been described in publication [c.f. (8) and personal communication with Dr. P. H. Coleman].

ologous V. An analogous gradient in specificity of protection could also be demonstrated by passive immunization, although at a lower level. In spite of fairly substantial protection after one immunization, no conventional HI, CF or N antibody could be detected, but (AGN) antibody was detected by an antiglobulin technique. Our results strongly suggest that the antiglobulin technique is a more sensitive technique for the detection of a neutralizing antibody, and, that the protection can be therefore correlated with such antibody. It seems worth noting, however, that antiglobulin can be used to cause or augment virus neutralization *in vitro* by "incomplete" (*i.e.* partially enzyme-digested) antibody or antibody complexed with virus which permits the virus to remain infectious [reviewed in (4)]. Granted that such antibodies are inefficient in neutralizing virus *in vitro*, it is nevertheless conceivable that, aided by humoral and/or cellular factors *in vivo*, they may account for the protection that was observed. However, the gradient in specificity that was observed, that is, differences in protection against E_m and E, would then need to be explained by differences in the virulence the two viruses and/or subtle differences in the binding of the antibodies to the two viruses, etc. A determination of K values was attempted to test the latter idea using the indirect AGN technique, but differences that were observed were not reproducible, perhaps because of the different kinetics involved in the "sandwich" AGN technique that employs two antigen-antibody reactions.

Because antibody was not detectable by conventional techniques in singly immunized mice that were subsequently protected against challenge by virulent virus, tests were made for non-specific interference, and for IF. These tests were carried out even though, 1. protection due to non-specific interference and/or IF usually occurs only early after immunization (7, 26), and 2. antibody was detected by the AGN technique in our experiments. The results of the non-specific interference tests showed that none was detected. Neither was IF detected in the same fluids that could passively protect mice against challenge.

Two immunizations with E_m, or with E_m and E (the latter data not shown), increased the level of protection and showed broadened cross protection. This cross protection was similar to that described by others for Group A (and other) arboviruses; the protection, for example, extended to V in spite of the fact that no cross neutralizing antibody against V was detected in our experiments, as well as those of others (6, 8, 15, 17). Even the antiglobulin test failed to show cross neutralizing antibody between E_m or E, and V viruses. Furthermore, in one of our unpublished experiments using fluids taken from doubly immunized mice, and independently in those of Hearn, H. J., Jr., [Fed. Proc. **21**, 461, (1962)], passive homologous protection against V virus was strong but cross protection between E and V could not be demonstrated as was shown among Group B arboviruses (23, 30). The latter cross protection was hypothesized to be due to a serum protective factor(s) independent of both antibody and interferon.

A specific humoral mechanism has been invoked to explain cross protection which is based on an unusually rapid (anamnestic-like) induction of N antibody to the second Group-related yet heterologous challenge virus in mice previously immunized with the first (6, 17). This explanation, however, has been challenged [Brand, O. & W. P. Allen, Bact. Proc. p. 126, 1964; (8, 23)]. We are also aware

of unpublished experiments in other laboratories in which failures were encountered in attempts to explain cross protection in the absence of N antibody on the basis of a cell-mediated immune response. In one of these (Allen, W. P., 1965, personal communication) one million spleen cells from a SF immunized animal conferred excellent homologous passive protection but failed to show cross protection against V virus. Whether a specific cell-mediated immune response recently shown in infection of mice with Sindbis or Venezuelan encephalitis virus (11, 24) can account for heterologous cross protection within the Group A arboviruses is unknown, but is now under study in our laboratory. In a similar vein, speculations concerning the role of defective interfering particles in protection, or possibly cross protection *in vivo* (18), have not proceeded to the experimental stage in arbovirus research, although defective interfering particles in the Group A arboviruses have been found recently in cell culture (27, 28).

Finally, it is perhaps pertinent to comment on whether E_m has potential as an attenuated vaccine in man for homologous and/or heterologous protection. The difficulties inherent in developing and testing an attenuated Group A arbovirus as a vaccine to be used in humans have been partially reviewed (*e.g.* 8). The fact that E_m retains a high level of virulence for mice by the IC route, and, a lower level of virulence, although erratically, by the IP route makes it an unlikely candidate for an attenuated vaccine for human use without further modification. However, E_m may deserve additional study to see if it can serve as a preferable alternate to other more virulent strains for use as a killed vaccine, or as a mutant to modify further for use as an attenuated strain in higher animals if not in man.

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