Resistance to Arbovirus Challenge in Mice Immediately After Vaccination

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Mice vaccinated with a single injection of formalin-inactivated suspensions of mouse brain tissue infected with arboviruses were markedly protected against a challenge injection administered hours later. The protection observed during the first 2 days after vaccination seemed to be nonspecific, in that it appeared not only with the homologous system but also between arboviruses of different antigenic groups; this phase may be associated with an interferon-like activity of the serum. Overlapping the nonspecific phase was one of specific protection, which seemed to be well-established in its own right by day 3 or 4 after vaccination. Serum neutralizing antibodies against the homologous viruses were detected as early as 24 h after vaccination and in almost all instances by day 3.

With certain virus infections of man, their universal distribution and the fact that the serotypes responsible are relatively few in number and antigenically either homogeneous or very similar, make preventive vaccination the accepted rule. Arbovirus infections, by contrast, are geographically and ecologically localized and represent numerous distinct serotypes; even if safe vaccines were available for all that cause human disease, general preventive vaccination would hardly be advisable. Vaccination against arboviruses would, however, be recommended in circumstances such as travel into an affected area, epidemic outbreaks appearing in an endemic zone, or extension of the virus in epidemic form into a new area. For all these situations, it is important to have information about the speed with which effective immunity develops (4).

This paper, which represents an extension of previous studies made over a number of years (5; D. W. Barry, M.D. thesis, Yale University School of Medicine, 1969), reports the results of experiments on the development of resistance in mice to arbovirus challenge immediately following vaccination.

MATERIALS AND METHODS

Mice. Mice derived from the Charles River CD(R)-1 strain were random bred in a barrier colony maintained at this laboratory.

Viruses. The following viruses and strains were used: Eastern equine encephalitis (EEE), strain CDC-Alabama 09-28-0930, 6th to 8th mouse passage; Semliki Forest (SF), Smithburn original strain, 20th to 26th mouse passage; Venezuelan equine encephalitis (VEE), strain Trinidad-1945, 10th to 12th mouse passage; Banzi, strain H-336, 6th to 9th mouse passage; Japanese encephalitis (JE), strain Nakayama, between the 100th and 200th mouse passage; West Nile (WN), strain IbAr 4029, 4th to 6th mouse passage; and Colorado tick fever (CTF), strain Condon, 36th to 38th mouse passage.

Virus stocks, consisting of 10% suspensions of infected newborn mouse brain tissue, were made in physiological saline with phosphate buffer, pH 7.2, containing 7.5% bovine plasma albumin. The suspensions were held in 0.5-ml amounts in sealed glass ampules at −70 °C in an electrically driven freezer.

Vaccines. Brain tissues of newly infected 2- to 4-day-old mice were made up as 10% suspensions in physiological saline; 0.5% commercial formalin was added and each suspension, in a glass-stoppered bottle, was kept at 4 °C for 7 days. (These freshly prepared suspensions were not generally titrated prior to addition of formalin. In our current and past experience, the infective titers of similar preparations, determined by intracerebral inoculation of 0.02 ml into 2- to 4-day-old mice, have been: for EEE, VEE, SF, and Banzi, between 10^4 and 10^6; for JE and WN, 10^4.4 and 10^4.4; and for CTF, 10^6.) The vaccines were then stored at −60 °C in plastic containers until used 1 to 6 months later.

Cell cultures. The Vero cell line and cell strain L (NCTC clone 929) were propagated and maintained as follows: Stock cultures were carried in Roux bottles (Vero cell line) or 3-oz flint-glass prescription bottles (cell strain L) with a growth medium consisting of Eagle minimal essential medium prepared with Hanks balanced salt solution (HBSS) and 10% fetal bovine serum. Stock cultures were transferred weekly.
by a 1:5 (Vero cell line) or 1:12 (cell strain L) split ratio. All media contained 100 units of penicillin and 100 μg of streptomycin per ml.

Vaccination and challenge. Adult mice were vaccinated once with 0.2 ml of vaccine, usually by the intraperitoneal (ip) route; the vaccines were used undiluted and, in a few instances, also in dilution 1:10. Vaccinations were done on different days, but all mice in a given experiment were challenged on the same day except in the first experiment. The challenge inoculum, 0.2 ml, was usually given subcutaneously (sc) in the inguinal region, with the mice under ether anesthesia because this prevented their struggling and thus promoted the speed and accuracy of the procedure.

Prior to the challenge experiments, the infective titers of the virus stocks were assayed in normal mice 30 to 60 days of age. With EEE, JE, and WN viruses, mice of the stated ages were not uniformly susceptible to sc inoculation of consecutive decimal dilutions from 10⁻¹ to 10⁻⁷ (Table 1). Accordingly, in challenge experiments with these three viruses, mice 30 to 35 days old were challenged with a dilution of virus expected from the assay to contain 10 to 100 times the dose present in the highest dilution that killed significant numbers of mice (30-60%) in a titration in controls. As SF, VEE, and Banzi viruses proved uniformly lethal on assay, in challenge experiments with these viruses, mice 50 to 60 days old were challenged with dilution estimated from the assay to contain 10 to 100 50% lethal doses (LD₅₀). In all experiments, additional smaller groups of control mice were challenged with two or three consecutive dilutions of the amount of challenge virus given the bulk of the animals. Table 2 shows the mortality caused among normal unvaccinated control mice in the various experiments by the virus dilution (a) selected for challenge.

Results of the challenge tests were evaluated on the basis of death or survival of the mice within the period of observation: 14 days for the viruses with short incubation, 28 days for those with longer incubation. The numbers of mice dead in the vaccinated groups were compared with the number dead in the unvaccinated control group by the chi square test in a 2 x 2 table, using Yates' correction for small numbers. With groups of 32 mice, a 35% difference in the mortality rates was significant at the p ≤ 0.05 level.

Mouse neutralization tests. These neutralization (N) tests were performed in 2- to 4-day-old mice inoculated ip with 0.06-ml volumes of virus-susceptible mixture. Each "serum" represented pooled sera obtained from a set of vaccinated or control mice that were bled once and discarded. Usually undiluted serum was mixed with an equal volume of virus, the latter in two consecutive decimal dilutions estimated to contain 10 and 100 LD₅₀. The mixtures were incubated for 2 h at 37°C before inoculation. Because of the occurrence of deaths due to cage contamination (sometimes the litter's mother also died), the tests were terminated as soon as the corresponding controls were all dead: on day 3 after inoculation (EEE, SF, VEE) or day 7 (Banzi, JE, WN).

A serum was considered positive if the log₁₀ neutralization index, estimated by the Reed-Muench method, was 2 or higher. When the amount of serum available was not sufficient for a complete titration, a serum was considered positive if two, one, or no mice died of eight inoculated with a mixture of that serum and from 50 to 200 LD₅₀ of virus.

Plaque reduction tests. Two-ounce flint-glass prescription bottles were seeded with 10 ml of a Vero cell suspension prepared by dispersing cells of one stock culture in 320 ml of growth medium. These cultures were used for plaque reduction (PB) tests 2 days after seeding. Serial twofold dilutions of serum (obtained as described for mouse N tests), beginning at 1:2, were mixed with an equal volume of a virus suspension containing an estimated 100 plaque-forming units (PFU) in 0.1 ml. After incubation at 37°C for 1 h, the mixture was inoculated in 0.2-ml amounts into two cultures from which the fluid medium had been removed. Each inoculum was adsorbed for 1 h at 36°C, after which 10 ml of a freshly prepared nutrient-agar overlay was added. The composition of the nutrients in the overlay was that described by Simizu et al. (13), as modified by Karabatsos (9); the final overlay mixture contained 100 μg of diethylaminomethyl dextran (DEAE dextran). The overlaid cultures were incubated in an inverted position at 36°C for 7 days; final readings of plaque counts were carried out at the end of that time or after an additional 7 days' incubation at room temperature (20-25°C).

The titer of a serum was expressed as the highest dilution of serum giving a 50% reduction in the plaque count (10).

Table 1. Susceptibility of mice to subcutaneous inoculation of EEE, JE, and WN viruses

| Virus* | Age of mice (days) | Dilution of virus (exponent to base 10) |
|--------|-------------------|---------------------------------------|
|        |                   | -2  | -3  | -4  | -5  | -6  | -7  | -8  |
| EEE    |                   | 27  | 16/16 | 12/16 | 14/16 | 12/16 | 13/16 | 8/16 | 2/16 |
|       |                   | 60a | 10/12 | 7/12 | 1/12 | 5/12 | 3/12 | 5/12 |      |
| JE     |                   | 30  | 4/7  | 5/7  | 7/7  | 4/7  | 7/7  |      |      |
|       |                   | 28  | 10/16 | 8/16 | 11/16 | 13/16 | 22/32 | 4/16 | 4/16 |
| WN     |                   | 36  | 6/16 | 7/16 | 13/16 | 8/16 | 13/16 | 4/16 | 1/16 |
|       |                   | 30  | 11/16 | 13/16 | 16/16 | 14/16 | 13/16 | 5/16 | 8/16 |

*Inoculum of 0.2 or 0.3 ml.
*a Mice dead per mice inoculated.
*a These mice were inoculated ip.
Table 2. Summary of mortality caused among normal unvaccinated control mice by the virus dilution(s) used in the challenge experiments

| Virus | Dilution (exponent to base 10) | Mice dead per mice inoculated |
|-------|-------------------------------|------------------------------|
| EEE   | -5 or -6                      | 19/32, 22/32                 |
| SF    | -7 or -8                      | 46/48, 39/40, 21/21, 21/21, 30/32 |
| VEE   | -8                            | 32/32, 32/32                 |
| Banzi | -8                            | 34/35                        |
| JE    | -5 or -6                      | 22/40, 23/40, 26/48, 13/32   |
| WN    | -5 or -6                      | 13/32, 30/32, 12/32          |

**Interferon assay.** Stationary tube cultures were prepared with 1 ml of a Vero cell suspension representing the cells of one Roux bottle harvested in 320 ml of outgrowth medium, or with 0.5 ml of an L strain cell suspension representing the cells of one 3-oz flint glass prescription bottle harvested in 6 ml of outgrowth medium; the medium consisted of Eagle basal medium (EBM) made up with HBSS, 10% tryptose broth, and 10% fetal bovine serum. The cultures were used after incubation for 2 to 3 days at 36 C.

Assay was done by the method of Baron (2). The pH of the sera was brought down to 2.2 by adding 1 N HCl, and the sera were kept at 4 C for 7 days; the pH was then adjusted back to 7 to 7.2 with 1 N NaOH and the sera were tested for ability to inhibit multiplication of vesicular stomatitis virus (VSV), type Indiana (American Type Culture Collection, used at the second to third passage level in this laboratory). Serum diluted 1:3 with maintenance medium was added to the tube cultures, three replicate tubes per sample. After incubation at 36 C for 20 h, the cultures were rinsed twice with 2 ml of HBSS and inoculated with 0.2 ml of virus at a multiplicity of 20 PFU per cell. The cultures were then reincubated at 36 C for 1 h, rinsed four times, and refed with a maintenance medium consisting of 97% EBM made up with HBSS plus 3% fetal bovine serum and adjusted with 0.2 to 0.4 ml of 2 M Trizma base (reagent grade; Sigma Chemical Co., St. Louis, Mo.) per 200 ml of medium. After further incubation at 36 C for 21 h, the fluids from the three tubes were collected, pooled, and stored at -60 C until titrated by plaque assay in Vero cells. Each assay included a sample of mouse interferon standard reagent supplied by the Research Reagents Branch, National Institutes of Health.

Results were evaluated by comparing the effect of the serum with that of the mouse interferon standard used in the same test in a series of dilutions containing 60, 20, 6, 2, and 0.6 units/ml. When a similar plaque count was obtained with a serum and with a given dilution of the interferon standard, it was considered that the two had equivalent activity in terms of number of interferon units contained.

**RESULTS**

**Resistance to homologous challenge.** In the initial experiments, the development of resistance following vaccination was investigated over a longer period than was later adopted. Table 3 shows the results of homologous challenge tests with SF virus.

For reasons of convenience the experiment was divided into four sections, each of which included a group of 21 unvaccinated control mice that were challenged with the same virus dose as the vaccinated animals, and additional mice to permit a virus titration. Resistance to a challenge of 500 LD50 of SF virus was observed from day 3 after vaccination onward (for day 4, 0.01 > p > 0.001). Neutralizing antibody was detected on day 3 and subsequent days.

Table 4 shows the development of resistance to homologous challenge with JE and Banzi viruses in the period of 3 to 4 days after vaccination. Even though only 13 of 32 normal mice challenged with JE virus (dilution 10^-4) died, all vaccinated mice survived, including those given the vaccine 24 h after being challenged. Neutralizing antibody was first detected in the serum on day 3 after vaccination. In the test with Banzi virus, solid protection against 30 LD50 of virus was noted 24 h after vaccination and later, and neutralizing antibody was present in the sera on days 1 through 4.

**Resistance to cross-challenge: nonspecific resistance.** Attempts were next made to determine whether nonspecific resistance to a challenge developed in the early days after vaccina-

Table 3. Development of resistance to homologous challenge and of neutralizing antibody in mice vaccinated with SF virus

| Challenge test i.p. | Virus i.p. N test* | Plaque reduction test* (PFU/0.2 ml) |
|---------------------|--------------------|-------------------------------------|
| Days after vaccina-  | Virus dilution 10^-4| Virus dilution                       |
| tion s.c.           | Vaccinated mice    | Control mice                        |
| 1                   | 19/21             | 21/21                               |
| 2                   | 22/21             | 21/21                               |
| 3                   | 12/21             | 21/21                               |
| 4                   | 13/21             | 21/21                               |
| 5                   | 7/21              | 21/21                               |
| 6                   | 3/21              | 21/21                               |
| 7                   | 4/21              | 21/21                               |
| 14                  | 1/21              | 21/21                               |
| 28                  | 0/21              | 21/21                               |

* The titer of virus in the test was 10^4. With eight control sera, 64/64 mice given dilution 10^-7 died and 63/64 mice given dilution 10^-4.

* Virus dilution, 10^-7. With the control serum, the result was 38 PFU/0.2 ml.

* Mice dead per mice tested.
tion. In these experiments, different sets of mice were vaccinated (i) with vaccines prepared with viruses antigenically unrelated to the challenge virus, (ii) with a normal mouse brain suspension treated with formalin in the same way as the virus vaccines, and (iii) with the homologous virus vaccine.

In a challenge test with EEE virus (Table 5, Fig. 1), EEE vaccine protected mice challenged at 6 h, 1 day, and 7 days after vaccination, but not at 2 days (14/32 mice died, chi square = 3.1, p > 0.05). JE vaccine protected against EEE virus challenge at 6 h and 1 day after vaccination, barely at 2 days (12/30 mice died, chi square = 4.07, p slightly under 0.05), and not at 7 days. Vaccination with a normal brain suspension failed to influence the outcome of challenge. The challenge dilution of EEE virus, 10^{-6}, killed 22 of 32 unvaccinated mice; in addition (not shown in the table), dilutions 10^{-4} and 10^{-7} each killed 5 of 8 unvaccinated mice.

In a challenge test with SF virus (Table 5, Fig. 2), undiluted SF vaccine protected throughout the 4-day period of study. SF vaccine in dilution 1:10 protected mice on days 1 and 4 but not on days 2 and 3. A CTF virus vaccine protected on day 1 only. The amount of virus in the challenge was approximately 3 LD_{50}; dilution 10^{-7}, used as challenge, killed 24 of 32 controls and dilutions 10^{-4} and 10^{-9}, 1 of 8 and 0 of 8, respectively.

Results of further cross-challenge tests are summarized in Table 6. Cross-protection was observed only when challenge was made 1 or 2 days after vaccination, or sometimes 1 day before. Even in the challenge tests with WN virus, which were barely significant or lacked statistical significance because of the low sus-

### Table 4. Development of resistance to homologous challenge and of neutralizing antibody in mice vaccinated with JE and Banzi viruses

| Vaccine virus | Challenge test s.c. | Plaque reduction test* (PFU/0.2 ml) |
|---------------|---------------------|------------------------------------|
|               | Virus dilution (exponent to base 10) |                                    |
|               | -6 | -7 | -8 | -9 | -10 |
| JE | -1 | 0/32* | 40 |
|    | 1/24 | 0/32 | 25 |
|    | 1 | 0/32 | 23 |
|    | 2 | 0/32 | 11 |
|    | 3 | 0/32 |                |
| Controls | 13/32 | 9/16 | 3/16 | 34, 40 |
| Banzi | 1 | 0/35 | 20 |
|    | 2 | 0/35 | 18 |
|    | 3 | 0/35 | 8 |
|    | 4 | 0/35 | 6 |
| Controls | 34/35 | 7/8 | 1/8 | 87 |

* Virus dilution, 10^{-6}.

* Mice dead per mice challenged.

### Table 5. Development of nonspecific resistance to homologous and heterologous challenge in mice after vaccination with EEE and SF viruses

| Challenge virus, dilution | Vaccine | Challenge test s.c., days after vaccination i.p. |
|--------------------------|---------|-----------------------------------------------|
|                         |         | 1/4 | 1 | 2 | 3 | 4 | 7 |
| EEE, 10^{-4} | EEE | 2/32* | 2/32 | 14/32 | 2/32 |
|               | JE | 4/31 | 2/32 | 12/30 | 20/32 |
|               | Normal brain | 16/32 | 27/32 | 21/32 |
|               | None | 22/32 |                |
| SF, 10^{-7} | SF, undiluted | 4/32 | 6/32 | 1/32 | 1/32 |
|               | SF, 1:10 | 11/32 | 19/32 | 16/32 | 6/32 |
|               | CTF | 9/32 | 23/32 | 21/32 | 21/32 |
|               | None | 24/32 |                |

* Mice dead per mice challenged.
RESISTANCE TO ARBOVIRUS IN MICE

Fig. 1. Resistance of mice to EEE virus challenge after vaccination with formalin-inactivated EEE virus, JE virus, and normal mouse brain tissue. Protection of mice expressed as [(percent dead controls − percent dead vaccinated)/percent dead controls] × 100.

Fig. 2. Resistance of mice to SF virus challenge after vaccination with formalin-inactivated SF virus, undiluted and in dilution 1:10, and Colorado tick fever virus, undiluted. Protection of mice expressed as in Fig. 1.

Table 6. Development of nonspecific resistance to homologous and heterologous challenge in mice after vaccination with SF, VEE, Banzi, JE, and WN viruses

| Test no. | Challenge virus, dilution (LD_{50}) | Vaccine | Challenge test s.c. |
|----------|----------------------------------|---------|---------------------|
|          |                                  |         | 1                   | 2 | 3 | 4 | 7 |
| 1        | SF, 10^{-4} (110)                | SF      | 8/40                | 0/40 | 0/40 |
|          |                                  | JE      | 32/40               | 27/40 | 29/32 |
|          |                                  | Normal brain | 36/40   | 39/40 | 26/32 |
|          |                                  | None    | 39/40               | 39/40 | 39/40 |
| 2        | VEE, 10^{-4} (100)               | VEE     | 2/32                | 0/32 | 0/32 | 0/32 |
|          |                                  | CTF     | 31/32               | 30/32 | 31/32 | 30/32 |
|          |                                  | Normal brain | 32/32   | 32/32 | 32/32 | 32/32 |
|          |                                  | None    | 32/32               | 32/32 | 32/32 | 32/32 |
| 3        | Banzi, 10^{-4} (2)              | Banzi, undil. | 3/32    | 0/32 | 0/32 |
|          |                                  | Banzi, 1:10 | 7/32    | 4/32 | 2/32 |
|          |                                  | SF, undil. | 3/32    | 14/32 | 18/32 |
|          |                                  | SF, 1:10 | 8/32               | 12/32 | 19/32 |
|          |                                  | None    | 19/32               | 19/32 | 19/32 |
| 4        | JE, 10^{-4} (10)                | JE      | 5/40                | 0/40 | 0/40 |
|          |                                  | SF      | 8/40                | 10/40 | 12/40 |
|          |                                  | Normal brain | 17/40   | 22/40 | 22/40 |
|          |                                  | None    | 22/40               | 22/40 | 22/40 |
| 5        | JE, 10^{-4} (100)               | JE      | 4/48                | 0/48 | 0/48 |
|          |                                  | SF      | 11/48               | 16/48 | 18/48 |
|          |                                  | Normal brain | 15/48   | 15/48 | 15/48 |
|          |                                  | None    | 26/48               | 26/48 | 26/48 |
| 6        | WN, 10^{-4} (1–2)               | WN      | 2/32                | 6/32 | 9/32 | 5/32 |
|          |                                  | SF      | 4/32                | 9/32 | 9/32 | 8/32 |
|          |                                  | None    | 13/32               | 13/32 | 13/32 |
| 7        | WN, 10^{-4} (1–2)               | SF      | 5/32                | 5/32 | 5/32 |
|          |                                  | Normal brain | 7/32    | 7/32 | 7/32 |
|          |                                  | None    | 13/32               | 13/32 | 13/32 |

*See Materials and Methods for estimation of LD_{50} in the challenge.
* Days after vaccination i.p.
* Mice dead per mice challenged. Italicized figures are significant at the p < 0.05 level.
ceptibility shown by the mice, there was an indication of nonspecific protection on day 1 after vaccination. Nonspecific protection was not noticed, however, against VEE virus.

Homologous protection, whether due to specific or nonspecific causes, was generally observed from day 1 after vaccination onward; in addition, protection against JE virus was seen when challenge was made 24 h before vaccination.

**Development of neutralizing antibodies.** Development of neutralizing antibodies was investigated in several experiments in addition to those already reported in Tables 3 and 4. The combined results are given in Table 7.

It is worth emphasizing that when challenge tests were carried out simultaneously with N tests, effective resistance to the challenge was demonstrated in all instances when neutralizing antibodies were detected. Resistance also was demonstrated, however, in a number of instances when neutralizing antibody was not found (Tables 3 and 4).

**Interferon-like antiviral effect of serum.** Table 8 gives the results of interferon assays with sera of mice bled 1 h and 1, 2, and 3 days after vaccination with JE vaccine; this vaccine completely protected mice against homologous challenge at the stated times (see Table 4). Only the serum taken 1 h after vaccination had a definite effect on the multiplication of VSV-Indiana virus; the effect of the serum taken 1 day after vaccination, while showing a positive trend, cannot be given an unqualified interpretation. The 1-h serum had no detectable neutralizing antibodies, while the day-3 serum did (Table 4).

Assays with sera from mice bled 1, 2, 3, and 4 days after vaccination with Banzi virus failed to show any interferon-like effect on multiplication of VSV-Indiana virus in L cells. Plaque counts were, respectively, 1.2, 1.3, 2.3, and 1.9 PFU/ml × 10^4. With normal serum the count was 1.2 × 10^4 and with dilution 10^{-4.5} of the NIH mouse interferon standard (equivalent to 2 units/ml), 0.08 × 10^4. In the homologous challenge experiment with Banzi virus (Table 4), all vaccinated mice survived and neutralizing antibodies were detected in the sera taken on all 4 days.

Table 9 shows the results of assays with sera from mice vaccinated with VEE and CTF viruses and bled 1, 2, and 3 days later (results of the challenge test with VEE virus are given in Table 6). In L cells, the sera from VEE-vaccinated mice alone had a significant and consistent interferon-like effect; in Vero cells, no interference was demonstrated even with these sera. VEE-vaccinated mice first had detectable serum neutralizing antibodies on day 3 after vaccination (Table 7).

**DISCUSSION**

Reasons have already been given as to why it is important to ascertain how soon after immunization with an arbovirus a host can reasonably be expected to be protected against a challenge with the same agent.
Detection of circulating antibody within a few days after immunization has been reported by various workers (3, 7, 11, 14, 15). Likewise, since the demonstration that resistance to homologous challenge was detectable 4 days after administration of a formalin-inactivated Russian spring-summer virus vaccine (6), observations of early resistance after vaccination have been extended to a number of other arboviruses (5; D. W. Barry, M.D. thesis, Yale University School of Medicine, 1969).

In mice vaccinated ip with live attenuated VEE virus, Hearn and Rainey (8) observed marked protection 24 h later against ip challenge with VEE and EEE viruses, and persistence of the protection for at least 60 days; in addition, shortlived protection developed against an antigenically unrelated virus, vaccinia. No cross-neutralizing antibodies were detected in the immediate period after vaccination. The authors concluded that the early phase of protection was nonspecific in nature and attributed it to interference by viral exclusion.

Price et al. (12) found that mice vaccinated ip with live Langat virus were protected against ip challenge with JE virus, of the same antigenic group, during the first 2 days after vaccination but not later. This early cross-protection, they concluded, was due to an interference-interferon mechanism. They state, however, that no interferon activity was present in the serum at the moment of challenge, and that while interferon developed at 6 h after challenge, its presence was not correlated with cross-protection.

More recently, early resistance to homologous challenge after vaccination has been observed in mice vaccinated with rabies virus, either live attenuated (16) or inactivated (1). In both instances, administration of the vaccine resulted in development of interferon.

In our experimental model, we deliberately choose (i) to use inactivated virus vaccines, so as to circumscribe the immunizing stimulus to a given amount and a precise time, and (ii) to use different routes for vaccination and challenge, so as to minimize the possibility of the vaccine and challenge viruses conflicting with each other topically in a somewhat artificial situation. With viruses that follow the same pathway, a live-vaccine virus may become latently established within strategically located cells and exclude the challenge virus (8).

The results reported show that resistance to a homologous challenge of between 1 to 3 and

| Serum, *mice vaccinated with | Days after vaccination | Test for interferon activity with VSV-Indiana virus |
|-----------------------------|------------------------|--------------------------------------------------|
|                             |                        | L cells (PFU/ml x 10^3) | Vero cells (PFU/ml x 10^3) |
|                             |                        | Count | Titer ratio | Count | Titer ratio* |
| VEE virus                   | 1                      | 0.16  | 15          | 0.5   | 6            |
|                             | 2                      | 0.05  | 46          | 1.3   | 2.4          |
|                             | 3                      | 0.015 | 153         |       |              |
| CTF virus                   | 1                      | 0.5   | 4.6         | 1.5   | 2            |
|                             | 2                      | 0.75  | 3           |       |              |
|                             | 3                      | 2.4   | 1           | 3.0   | 1            |
| Normal mouse brain          | 1                      | 1.05  | 2.3         | 0.8   | 3.9          |
|                             | 2                      | 0.6   | 3.8         | 2.4   | 1.3          |
|                             | 3                      | 1.15  | 2           | 0.8   | 3.9          |
| Controls (unvaccinated normal mice): |            |        |              |        |              |
| Serum 1                     |                        | 1.4   | 1.6         | 2.0   | 1.6          |
| Serum 2                     |                        | 0.9   | 2.5         |       |              |
| Serum 3                     |                        | 2.8   | 0.8         |       |              |
| Serum 4                     |                        | 2.3   | 1           |       |              |
| VSV-Indiana virus titer     |                        | 2.3   |             | 3.1   |              |
| NIH mouse interferon:       |                        |       |              |       |              |
| 10^-4 (60 units/ml)         |                        | 0.003 | 770         | 3.2   | 1            |
| 10^-5 (6 units/ml)          |                        | 0.028 | 82          | 3.4   | 0.9          |

* Sera assayed at dilution 1:3.
* Expressed as the reciprocal of the ratio between the PFU/ml count for a given sample and that of the virus with no serum.
100 LD₅₀ was established within 1 to 24 h after a single injection of formalin-inactivated vaccine; indeed, with challenge viruses having a long incubation period, protection also was afforded by vaccination 24 h after challenge. In some instances, specific neutralizing antibodies were detected 24 h after vaccination, and whenever antibodies were found the mice were protected against challenge. Occasionally, this early protection was noted in the absence of detectable neutralizing antibodies; furthermore, it was nonspecific in that it occurred between antigenically unrelated viruses and could even be brought about by formalinized normal mouse brain tissue suspensions.

An interferon-like effect was detected with sera taken in the immediate period after vaccination. This effect did not disappear when the sera were held at pH 2 for several days; it was seen in homologous (mouse) L cells but not in Vero cells; and it was active against an antigenically unrelated virus. Although development of interferon following administration of inactivated or nonreplicating viruses is well known, the same is not true for interferon production following administration of arboviruses inactivated by such drastic means as formalin at a 0.5% concentration.

Whether the interferon-like effect of the serum was partially or totally responsible for the early protection observed cannot be stated as yet; the serum protective factor (neither antibody nor interferon) investigated by Price et al. (12) also deserves consideration. Whatever the mechanisms involved, following vaccination with formalin-inactivated vaccines a two-phase protection was discernible in our tests, most notably in experiments in which a diluted vaccine protected on day 1 after vaccination, not on day 2, but again on day 7, with the earlier protection occurring between antigenically unrelated viruses and the later only with the homologous system. That the two phases may, in addition, overlap is clearly indicated by the challenge test results with VEE virus.

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