Immunity to Aerosol Challenge in Guinea Pigs Immunized with Gamma-Irradiated Venezuelan Equine Encephalitis Vaccines

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In a previous report, it was shown that nonviable Venezuelan equine encephalitis (VEE) vaccines prepared by exposure of virus suspensions produced in WI-38 cells to ionizing radiations were highly effective in protecting guinea pigs subjected to intraperitoneal (ip) challenge with VEE virus. To characterize further the efficacy of irradiated vaccines, guinea pigs were immunized with three lots of vaccine inactivated by exposure to $8 \times 10^4$ r of gamma rays and then were challenged via the respiratory route with aerosols of VEE virus. Animals that received a series of three ip inoculations of vaccine at 1-week intervals showed high levels of resistance to aerosol challenge. The 50% effective dose values of vaccines ranged from <0.0016 to 0.0051 ml for respiratory challenge and from <0.00074 to 0.0011 ml for intraperitoneal challenge. Serological studies showed that antigenicity of the irradiated vaccines was excellent. Moderate to high levels of serum-neutralizing and hemagglutination-inhibiting antibodies were demonstrated in the majority of animals vaccinated with undiluted or 10-2 dilutions of the vaccines. However, serum-neutralizing and hemagglutination-inhibiting antibody levels were not always indicative of the level of immunity, because some animals in which significant antibody could not be demonstrated were able to survive challenge with VEE virus.

Although Venezuelan equine encephalitis (VEE) is spread from mammal to mammal or bird to mammal primarily by the bite of an infected mosquito, infections have been reported (10) to occur via the respiratory tract in humans and by contact in horses (9). The hazard of aerosols to laboratory workers who perform studies with infectious viruses is well documented (16). Protection of the laboratory worker by immunization with effective vaccines is desirable for investigations with hazardous microbes. Although a Formalin-killed VEE vaccine and a live attenuated vaccine have been tested in man, the former has been found to be ineffective for prevention of infection (11) and the latter is associated with side reactions in approximately 20% of the vaccinees (P. J. Kadull, personal communication). Recently one of us (M.R.) reported on the development of nonviable VEE vaccines by exposure of virus suspensions to gamma radiations (15). These vaccines have been shown to stimulate high levels of protection in vaccinated guinea pigs and mice against intraperitoneal (ip) challenge with the virulent Trinidad donkey brain strain of VEE virus (6). It was of interest to investigate the effectiveness of an irradiated VEE vaccine against challenge via the respiratory route. This report presents data on the responses of guinea pigs vaccinated with an irradiated VEE vaccine to aerosol challenge with VEE virus.

MATERIALS AND METHODS

Irradiated VEE vaccine. Three lots of irradiated vaccine were prepared from suspensions of VEE virus grown in human diploid cell monolayers (WI-38) as described previously (15). Seed virus used for preparation of vaccines had been passaged 14 times in chick embryos and once in WI-38 cell monolayers. Vaccines were prepared from the culture fluids of WI-38 monolayers infected with the seed virus and were inactivated by exposure to $8 \times 10^4$ r of $^{60}$Co. Vaccine lots 59/2a, 59/2b, and 59/2d had preinactivation titers of 9.1, 9.3, and 8.9 mouse intraperitoneal 50% lethal doses (MIPLD50) per ml, respectively.

Immunization of guinea pigs. The antigen extinction test of Cole and McKinney (2) was used to assay the potencies of the vaccines. Guinea pigs (250 to 350 g) were divided into groups and vaccinated ip with 0.2-ml amounts of graded dilutions of vaccine at intervals of 1 week for a total of three injections. Dilutions were made in beef heart infusion broth.
Control groups consisted of (i) animals that did not receive vaccinations and (ii) animals that were vaccinated with irradiated tissue culture medium from virus-free monolayers.

**Calculation of 50% effective dose.** The potency of the vaccine is expressed as the 50% effective dose ($ED_{50}$), the quantity of undiluted vaccine inoculated per dose that protected 50% of the test animals. The dose that protects 50% of the test animals was calculated by the method of Reed and Muench (13). $ED_{50}$ values were calculated for aerosol and ip challenge. Thirty-two to 40 animals per group were tested by aerosol and 15 animals per group were tested by ip challenge: $ED_{50} = \text{volume of vaccine inoculated per dose (milliliters)} / \text{dilution of vaccine protecting 50\% of test animals.}$

**Aerosol challenge.** Twenty-three to 24 days post-vaccination, animals were challenged with the Trinidad donkey brain strain of VEE virus. Challenge doses are listed in the text for individual experiments.

**Aerosolization.** Aerosol of VEE virus were produced in a 115,000-liter static aerosol chamber (17; F. E. Ray, Abstr. 136th Meet. Amer. Chem. Soc., p. 27, 1959) employing an atomizer test fixture disseminating device (reference 8, p. 287). The air in the chamber was preconditioned to 75 F (24 C) and 80% RH. Desired aerosol concentrations, and thus guinea pig inhaled doses, were achieved by adjusting the amount of virus preparation disseminated and by diluting the aerosol with clean air adjusted to chamber conditions.

**Aerosol sampling.** Samples of aerosols were collected in AGI 30 impingers fitted with preimpingers (3, 17) and operated for 1 min at a collection rate of 12.5 liters/min. Impingement fluid (20 ml) was Sorensen buffer with egg yolk and AntiFOAM A [0.025% KH$_2$PO$_4$, 0.267% Na$_2$HPO$_4$, distilled water; autoclaved at 15 psi for 15 min and then 0.1% AntiFOAM A (Dow-Corning Corp., Midland, Mich.)] and yolk from 7-day-old embryonated chicken eggs at a ratio of one yolk to four buffer added]. Samples of aerosol were taken at the beginning and end of animal exposure periods to determine infectious units of VEE virus per liter of aerosol. This was achieved by diluting impingement fluid to appropriate dilutions in Heart Infusion Broth (Difco) and titrating intracerebrally in 10- to 14-g Swiss-Webster albino mice of mixed sexes to obtain 50% lethal dose ($LD_{50}$) values (13). Inhaled doses were calculated on the basis of aerosol concentrations, durations of exposures, and the guinea pig respiratory volume (5; reference 8, p. 295, 307). AGI 30 aerosol samplers collect droplet nuclei of about 5 µm or less in diameter and thus provide an estimate of that fraction of the aerosol inhaled by guinea pigs in the size range effective for initiating respiratory infection (1, 4, 7, 12). At the time of aerosol exposure, about 40 to 60% of the aerosol was composed of droplets 5 µm or less in diameter.

**Aerosol exposure.** Control animals and randomly selected guinea pigs from groups consisting of various vaccination levels were placed in perforated containers that were then suspended in the midst of the aerosol. Animals, therefore, were thus relatively unrestrained while being whole-body-exposed; this should have minimized or negated any effect on the normal breathing pattern (reference 8, p. 300).

**Serological assay.** Ten per cent or more of the animals were bled intracardially before vaccination, 21 days postvaccination, and 14 days postchallenge. Serum samples were assayed for hemagglutinating-inhibiting (HI; reference 14) and serum-neutralizing (SN) antibodies as previously described (15). SN indexes are expressed as log$_{10}$; HI titers are expressed as the reciprocal.

### RESULTS

**Efficacy of irradiated vaccines against aerosol challenge.** The initial experiment was designed to determine the response of immunized animals to various doses of the challenge virus. Guinea pigs received mean inhaled doses of approximately $6.5 \times 10^4$, $6 \times 10^4$, $1.6 \times 10^4$, and $1.6 \times 10^4$ MICLD$_{50}$. These animals had been vaccinated with lot 59/2a. Table 1 shows that this vaccine was highly effective against aerosol challenge and that no significant difference was apparent in the $ED_{50}$ values obtained, which ranged from 0.004 to 0.006 ml.

In the next two experiments, animals were vaccinated with lot 59/2b and lot 59/2d and then challenged with respiratory doses of approximately $3 \times 10^4$ to $7 \times 10^4$ and $3 \times 10^4$ to $6 \times 10^4$ MICLD$_{50}$ of VEE virus. In the previous experiment, as well as the next two experiments, the vaccinated guinea pigs unexpectedly did not demonstrate graded death responses to the graded aerosol challenge levels. In the interest of clarity, therefore, it was considered appropriate to evaluate their responses pooled over dose levels to demonstrate better the main experimental effects (namely, vaccine efficacy). Table 2 shows that animals vaccinated with undiluted and $10^{-1}$ dilutions of vaccine had high levels of SN and moderate levels of HI antibodies. These animals were also highly resistant to fatal infection with VEE virus.

Table 3 shows the results of HI antibody titrations of serum samples obtained in the final

| Inhaled dosage (MICLD$_{50}$) | $ED_{10}$ (ml) |
|-------------------------------|---------------|
| $6.5 \times 10^4$              | 0.0040        |
| $6.0 \times 10^4$              | 0.0044        |
| $1.6 \times 10^4$              | 0.0060        |
| $1.6 \times 10^3$              | 0.0058        |

* Lot 59/2a.
experiment before and after challenge. These data are typical of those obtained in the other experiments. Serum samples taken 14 days after aerosol challenge contained higher levels of SN and HI antibodies than prechallenge samples. Titers of HI antibodies were more elevated in animals challenged with higher doses of VEE virus than in those that received low doses. No correlation is apparent between the concentration of vaccine used for immunization and the postchallenge level of antibody response in animals challenged with $>10^2$ LD$_{50}$ doses. Although there was some increase in HI antibody levels in animals challenged with $>10^3$ LD$_{50}$, the response was not as marked in animals that received vaccine diluted as high as 1:100. In this experiment, although seven of the animals vaccinated with undiluted vaccine did not have significant HI titers ($<1:10$) at the time of challenge, none became ill.

Table 4 lists the antibody levels of five animals that responded poorly to vaccination. These animals, however, were able to survive challenge and produced high levels of HI and SN antibodies 2 weeks postchallenge.

Table 5 shows that the majority of paired serum samples tested showed a fourfold or greater increase in HI titer after challenge. Only 4 of 47 animals failed to have a significant increase in

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**Table 2. Immune response of vaccinated guinea pigs to challenge with VEE virus**

| Vaccine dilution | Prechallenge | | Prechallenge | | Prechallenge |
|------------------|--------------|------------------|--------------|------------------|--------------|
|                   | Per cent survival | HI | Log SN1 | Per cent survival | HI | Log SN1 | Per cent survival | HI | Log SN1 |
| $10^4$            | 94            | 49$^a$           | 4.6$^a$    | 98              | 38            | 6.3              | 100            | 30            | <3.4       |
| $10^{-1}$         | 91            | 34              | >2.6       | 81              | 30            | 6.5              | 72             | 15            | 3.1        |
| $10^{-2}$         | 26            | 5               | 0.9        | 68              | 5             | 2.2              | 63             | 6             | 1.5        |
| $10^{-3}$         | ND$^f$        | ND              | ND         | ND              | ND            | ND               | 14             | 1             | 0          |

$^a$ ED$_{50}$ values (milliliters, arithmetic mean) were as follows: vaccine lot 59/2a (1,600 to 654,000 MICLD$_{50}$), 0.0051 (aerosol), 0.0011 (ip); vaccine lot 59/2b (6,500 to 707,000 MICLD$_{50}$), <0.001 (aerosol), <0.00074 (ip); vaccine lot 59/2d (3,500 to 322,000 MICLD$_{50}$), 0.0017 (aerosol), 0.00072 (ip).

$^b$ Aerosol dose range.

$^c$ Lots 59/2a and 59/2b, 40 animals challenged per group; lot 59/2d, 32 animals challenged per group.

$^d$ Reciprocal of titer (geometric mean).

$^e$ Average.

$^f$ Not done.

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**Table 3. HI antibody titers in VEE-vaccinated guinea pigs before and after challenge with VEE virus via the respiratory route**

| Treatment               | Vaccine$^a$ dilution | <10$^b$ | 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | 5,120 | 10,240 | 81,920 |
|-------------------------|-----------------------|---------|----|----|----|----|-----|-----|-----|-------|-------|-------|--------|--------|--------|
| Postimmunization        | $10^{-2}$             | 2       | 5  | 7  | 3  | 4  | 5   | 1   |     |       |       |       |        |        |        |
|                         | $10^{-1}$             | 4       | 7  | 9  | 8  | 1  | 1   |     |     |       |       |       |        |        |        |
|                         | $10^{-2}$             | 12      | 10 | 7  | 2  |     |     |     |     |       |       |       |        |        |        |
|                         | $10^{-3}$             | 27      |     |     | 1  |     |     |     |     |       |       |       |        |        |        |
| Postchallenge ($>10^4$ MICLD$_{50}$) | $10^{-2}$             | 2       | 4  | 4  | 5  | 1  | 1,318 |     |     |       |       |       |        |        |        |
|                         | $10^{-1}$             | 3       | 2  | 3  | 2  |     | 3,373 |     |     |       |       |       |        |        |        |
|                         | $10^{-2}$             | 1       | 3  | 4  | 1  |     | 4,732 |     |     |       |       |       |        |        |        |
|                         | $10^{-3}$             | 1       | 2  | 2  | 2  |     |     |     |     |       |       |       |        |        |        |
| Postchallenge ($>10^3$ MICLD$_{50}$) | $10^{-2}$             | 1       | 1  | 3  | 1  | 4  | 1   | 1   | 2   |       |       | 189   |        |        |        |
|                         | $10^{-1}$             | 2       | 2  | 1  | 3  | 1  | 3   | 1   | 3   |       | 409   | 507   |        |        |        |
|                         | $10^{-2}$             |       | 5  | 3  | 1  |     |     |     |     |       |       | 1,687 |        |        |        |

$^a$ Lot 59/2d.

$^b$ Reciprocal of highest serum dilution which produced inhibition of hemagglutination.
TABLE 4. Immune response of vaccinated\(^a\) guinea pigs with prechallenge nonsignificant antibody levels

| Guinea pig | HI Pre-challenge | Post-challenge | Log SN1 Pre-challenge | Post-challenge | Aerosol challenge (MICLD\(_{50}\)) | 10\(^a\) | 10\(^b\) |
|------------|-----------------|---------------|-----------------------|---------------|---------------------------------|-------|-------|
| 119        | 10\(^b\)        | 320           | 1.0                   | 7.5           | S\(^a\)                          |       |       |
| 98         | <10             | 2560          | 1.2                   | 7.5           | S                               |       |       |
| 102        | <10             | 320           | 1.3                   | 7.5           | S                               |       |       |
| 103        | <10             | 5120          | 1.1                   | 7.5           | S                               |       |       |
| 106        | <10             | 320           | 1.1                   | 7.5           | S                               |       |       |

\(^a\) Animals vaccinated with 10\(^{-2}\) dilution of lot 59/2b.
\(^b\) Reciprocal of serum dilution.
\(^c\) Survived challenge.

TABLE 5. Number of VEE-vaccinated\(^a\) guinea pigs with fourfold rise in HI titer after aerosol challenge

| Vaccine dilution | Challenge dose | 10\(^a\) MICLD\(_{50}\) | 10\(^b\) MICLD\(_{50}\) |
|------------------|----------------|------------------------|------------------------|
| 10\(^a\)         | 4/7\(^b\)      | 8/8                    |                        |
| 10\(^{-1}\)      | 10/11          | 10/10                  |                        |
| 10\(^{-2}\)      | 6/6            | 4/4                    |                        |
| 10\(^{-3}\)      | 1/1            | NS\(^c\)               |                        |

\(^a\) Lot 59/2d.
\(^b\) No. of animals with fourfold rise in antibody titer/no. of animals challenged.
\(^c\) No survivors.

antibody level. Three animals had received undiluted vaccine and one had received a 10\(^{-1}\) dilution of vaccine; they were challenged with approximately 10\(^{3}\) MICLD\(_{50}\). All control animals died. No VEE antibodies were found in their prechallenge serum.

Reactions to vaccination. Guinea pigs were weighed and their rectal temperatures were recorded daily with an electronic thermometer (Tri-R Electronic Thermometer, Tri-R Instruments, Jamaica, N.Y.) starting 1 week before vaccination and continuing until 1 week post-vaccination. The animals continued to gain weight, and no significant increases in temperature were observed. No animals died as a result of vaccination with irradiated-inactivated virus.

DISCUSSION

Gamma-irradiated VEE vaccines were highly effective against respiratory challenge with virulent VEE virus. Immunized guinea pigs were able to resist aerosol doses that contained 47,127 times the inhaled LD\(_{50}\) for control animals.

Higher ED\(_{50}\) values for VEE vaccines were obtained when the challenge dose was administered by the aerosol route instead of the ip route. This may be assumed to be due to the fact that the guinea pig is less able to resist infection when the virus is administered by the aerosol route. This is supported by the data showing that the majority of the immunized animals became hyperimmune after challenge with aerosol doses as low as 10\(^{-4}\). Previous studies (15) have shown that this did not occur with guinea pigs challenged ip until doses as high as 10\(^{-4}\) were reached. Stimulation of antibodies by exposure to the higher doses of virus might be attributed to an anamnestic response to the antigenic mass; this is not true with the lower doses. In these animals, it may be assumed that some multiplication of virus particles occurred that stimulated the antibody-producing apparatus of the animal. However, it is apparent that the infection was quickly contained as symptoms of illness did not occur. Irradiated VEE vaccines were well tolerated by the guinea pigs. No side reactions were apparent in immunized animals which remained afebrile and continued to gain weight throughout the period of immunization.

Data presented indicate that gamma-irradiated VEE vaccines produced in WI-38 cells are safe and very effective for protection of guinea pigs against aerosol challenge. This type of vaccine also might well offer substantial protection to laboratory workers who may be exposed accidentally to aerosols or inoculation with virulent VEE virus.

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LITERATURE CITED

1. Casaret, L. J. 1960. Some physical and physiological factors controlling the fate of inhaled substances. II. Retention. Health Phys. 2:379.
2. Cole, F. E., Jr., and R. W. McKinney. 1969. Use of hamsters for potency assay of Eastern and Western equine encephalitis vaccines. Appl. Microbiol. 17:927-928.
3. Cown, W. B., T. W. Kethly, and E. L. Fincher. 1957. The critical orifice liquid impinger as a sampler for bacterial aerosols. Appl. Microbiol. 5:119-124.
4. Fothergill, L. D. 1957. Symposium on microbial aerosols and respiratory infection. Bacteriol. Rev. 21:249-250.
5. Guyton, A. C. 1947. Measurement of the respiratory volume of laboratory animals. Amer. J. Physiol. 150:70-77.
6. Hardy, F. M. 1959. The growth of Venezuelan equine encephalomyelitis virus in various tissue cultures. Amer. J. Hyg. 78:21-27.
7. Hatch, R. 1959. Respiratory dust retention and elimination, p. 113-132. In Proc. Pneumonoconiosis Conf., Johannesburg. Little, Brown and Co., Boston.
8. Jemuel, J. V., and G. B. Phillips. 1965. Aerosol challenge of animals, p. 273-341. In W. J. Gay (ed.), Methods of animal experimentation. Academic Press Inc., New York.
9. Kiasling, R. E., R. W. Chamberlain, D. B. Nelson, and D. D.
Stamm. 1956. Venezuelan equine encephalomyelitis in horses. Amer. J. Hyg. 63:274–287.
10. Lennette, E. H., and H. Koprowski. 1945. Human infection with Venezuelan equine encephalomyelitis virus. A report of eight cases of infection acquired in the laboratory. J. Amer. Med. Ass. 123:1088–1095.
11. McKinney, R. W., T. O. Berge, W. D. Sawyer, W. D. Tigertt, and D. Crozier. 1963. Use of an attenuated strain of Venezuelan equine encephalomyelitis virus for immunization of man. Amer. J. Trop. Med. Hyg. 12:597–603.
12. Morrow, P. E. 1960. Some physical and physiological factors controlling the fate of inhaled substances. I. Deposition. Health Phys. 2:336.
13. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493–497.
14. Reitman, M., and L. Green. 1970. Growth of Venezuelan equine encephalomyelitis virus in human diploid cell strain WI-38. Appl. Microbiol. 19:196–198.
15. Reitman, M., H. R. Tribble, Jr., and L. Green. 1970. Gamma-irradiated Venezuelan equine encephalitis vaccines. Appl. Microbiol. 19:763–767.
16. Reitman, M., and A. G. Wedum. 1956. Microbiological safety. Pub. Health Rep. 71:659–665.
17. Wolfe, E. K., Jr. 1961. Quantitative characterization of aerosols. Bacteriol. Rev. 25:194–202.