Gamma-Irradiated Venezuelan Equine Encephalitis Vaccines

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The efficacy of Formalin-inactivated Venezuelan equine encephalitis (VEE) vaccine has been reported to be low for man. Although a live VEE vaccine has been shown to be highly effective for the protection of laboratory workers, local and systemic reactions have occurred in approximately 20% of inoculated individuals. Therefore, studies were initiated in an attempt to produce an inactivated vaccine of high potency with low toxicity. Inactivated VEE vaccines were prepared by exposing virus suspensions to $8 \times 10^6$ or $10 \times 10^6$ r of gamma radiation. Irradiated VEE vaccines prepared from virus suspensions produced in Maitland-type chick embryo (MTCE) cell cultures and in monolayer cultures of human diploid strain WI-38 cells were highly immunogenic for mice and guinea pigs. Guinea pigs vaccinated with a series of three inoculations of vaccine (MTCE) survived challenge with at least $10^8$ mouse intracerebral 50% lethal doses of VEE virus. Irradiated vaccines induced high levels of serum-neutralizing and hemagglutinin-inhibiting antibodies in guinea pigs and rabbits. These findings suggest that ionizing radiation may be effective in the preparation of an inactivated VEE vaccine.

The preparation of inactivated vaccines by exposing suspensions of microorganisms to ionizing radiation has been reported for bacteria (5) and viruses (18). The X rays and gamma rays that are used for inactivation are short wavelength electromagnetic radiations that have high penetrating ability and have the desired characteristic of not imparting radioactivity to the exposed material. Studies with bacterial and animal viruses have shown that infectivity of viruses may be selectively destroyed by radiation while leaving antigenicity intact (4, 7, 11).

Unlike chemical treatment, which continues to act on the antigenic material in vaccines until the chemical is either removed or neutralized, the effect of radiation ceases upon completion of the exposure process.

Two types of Venezuelan equine encephalitis (VEE) virus vaccines have been produced and tested in man. A 0.4% formalinized chick embryo vaccine was prepared by Randall et al. (12) and has been used to vaccinate laboratory workers. Although this vaccine passed safety tests in guinea pigs, 14 human infections occurred in inoculated individuals (17). Repeated attempts by investigators in three laboratories to isolate virus from the various lots of vaccine under suspicion, by inoculation of the vaccine into a large number of mice, guinea pigs, monkeys, rabbits, “wet” chicks, and embryonated eggs, were unsuccessful. The authors suggested that man may be a more sensitive test organism for the detection of live VEE virus. A safe Formalin-inactivated vaccine was then prepared by Smith et al. (Bacteriol. Proc., p. 59, 1954) by treatment of the virus suspension with 4% Formalin. In a study conducted by McKinney et al. (8), 75% of individuals vaccinated with the latter vaccine developed a significant rise in hemagglutinin-inhibition (HI) and serum-neutralization (SN) antibody titers when inoculated with an attenuated VEE virus strain. The authors believed that the quantity of the antigen contained in the inoculum was too small to account for the response obtained and concluded that the killed VEE vaccine did not afford immunity to infection with the attenuated virus.

An effective live VEE vaccine has been prepared in fetal guinea pig heart cell monolayers (1). This vaccine has been reported to protect vaccinees against challenge with virulent VEE virus (8) and currently is being used to immunize laboratory workers at Fort Detrick. A single inoculation of $5 \times 10^7$ guinea pig intraperitoneal (IP) median immunizing doses stimulated the production of significant amounts of HI antibodies in approximately 96% of the vaccinees (R. W. McKinney, personal communication).
During the 6 years the vaccine has been in use, no clinical cases have been identified in those individuals who demonstrated a serological response to the vaccine. However, among vaccinated employees working in VEE-risk environments, a routine serological surveillance program has identified cases of VEE infection in three individuals who did not demonstrate an adequate response to vaccination with the live vaccine. These cases have been associated with presumed or documented accidental exposure to VEE virus and, in retrospect, had mild clinical symptoms compatible with VEE infection. Reactions associated with the viable vaccine have been recorded in approximately 20% of the vaccinees; symptoms reported are similar to those characterizing grippal or influenza-like syndromes (P. J. Kadull, personal communication).

In view of the side reactions associated with the use of the live vaccine and the poor potency of Formalin-inactivated vaccines, the present study was undertaken in an attempt to produce an inactivated VEE vaccine with potency approaching that of the viable vaccine but associated with a lesser reaction potential in humans. This report presents the preliminary data in animals oriented toward the development of such a vaccine.

**MATERIALS AND METHODS**

**Virus.** The Trinidad donkey brain strain of VEE virus (6) was obtained at this laboratory as a 10% chick embryo (12th passage) suspension in Beef Heart Infusion (BHI) broth. Seed virus was prepared from the 13th and 14th egg passages by inoculating 10*4* mouse intracerebral 50% lethal doses (MICLD50) of the virus into 10-day embryonated eggs via the allantoic route. The infected embryos were harvested after 24 hr of incubation at 35 C, and a 15% embryo suspension was prepared in BHI broth containing 400 units of penicillin and 400 µg of streptomycin per ml. Virus seed was stored in sealed ampoules at -70 C in a mechanical freezer.

**Irradiation.** Virus suspensions were irradiated with a 50,000-c cobalt* source at the National Bureau of Standards, as described in a previous communication (14). Irradiation doses are expressed as total doses in roentgens.

**Titration of virus suspensions.** Infectivity titers of virus were determined by intracerebral (IC) and IP inoculation of 0.03 ml and 0.2 to 0.25 ml, respectively, in 10- to 14-g Swiss mice, and by plaque assay in mouse fibroblast strain L cells. The 50% lethal dose end points were calculated by the method of Reed and Muench (13).

**Preparation of vaccines.** Tissue culture virus suspensions were prepared in chick fibroblast monolayers, in Maitland-type chick embryo suspensions (MTCE) as described previously (15), and in monolayers of WI-38 cells (16). Virus suspensions were clarified by centrifuging at 700 x g in a PR-2 (I.E.C.) refrigerated centrifuge. Two lots of vaccine were partially purified by differential centrifugation. The virus suspension was centrifuged in a Spinco model L ultracentrifuge at 8,720 x g for 15 min to remove debris; the supernatant fluid was then centrifuged at 54,500 x g for 60 min, and the virus pellet was resuspended in maintenance medium. Virus suspensions were distributed into serum bottles that were then sealed with rubber caps, frozen at -70 C, and irradiated by irradiation in the frozen state. Vaccines were kept at -70 C in a mechanical freezer until assayed.

**Safety tests.** Irradiated vaccines were tested for residual live virus by challenging five litters of suckling mice and by the antigenic capacity of a single inoculation in the guinea pig as described previously (14). No residual live virus was detected in virus suspensions exposed to 8 x 10*6* r or greater. Bacterial sterility of irradiated vaccines was determined by inoculation of liquid Thioglycollate Medium (Difco), Sabouraud dextrose agar, and nutrient agar.

**Vaccine assay.** The 50% effective dose (ED50) of the vaccines was determined by the method of Cole and McKinney (2) by challenging animals IP with approximately 10*4* MICLD50 of the homologous strain of VEE virus 21 days after administration of the last dose of vaccine. The ED50 is defined as that quantity of undiluted vaccine given in each dose of the series that protects 50% of the animals from death after challenge. Irradiated vaccines were thawed in a 37 C water bath just prior to inoculation of 10- to 40-g male mice (Swiss), or 250- to 350-g Hartley strain guinea pigs of both sexes. The animals received one, two, or three IP inoculations of vaccine spaced at intervals of 1 week. Inoculations were administered in amounts of 0.2 or 0.25 ml and were scheduled to allow for completion of the various series at the same time. Thus, animals immunized with one inoculation received their inoculation at the same time that animals immunized with three inoculations were administered their final inoculation.

Antibody levels were determined by SN and HI tests with prevaccination and postvaccination sera. The SN test described by Smith et al. (17) was employed with male mice. The challenge virus was a 15% suspension of VEE-infected chick embryo in BHI broth. The neutralizing index of the sera is expressed as log10. HI tests were performed as previously described (15) with an irradiated VEE hemagglutinin; the titers are expressed as the reciprocal.

**RESULTS**

Potencies of irradiated vaccines produced in avian and mammalian tissues. Preliminary assays of the potencies of irradiated preparations were performed in mice. Table 1 lists the ED50 values obtained with experimental vaccines prepared from virus suspensions with prevaccination titers ranging from 10*4* to 10*9* MICLD50/ml of infective virus.

Table 2 shows that the vaccines tested in guinea pigs had satisfactory potency values when administered in a series of two or three inoculations.
TABLE 1. Potencies of irradiated Venezuelan equine encephalitis vaccines for mice

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Cell culture prep</th>
<th>Preirradiation titer (log_{10} MLD_{50} / ml)</th>
<th>Radiation dose (r) X 10^6</th>
<th>2 Inoculations</th>
<th>3 Inoculations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR5</td>
<td>CF</td>
<td>8.1</td>
<td>8</td>
<td>0.062</td>
<td>0.0062</td>
</tr>
<tr>
<td>MR15</td>
<td>MTCE</td>
<td>10.0</td>
<td>10</td>
<td>0.01</td>
<td>0.0051</td>
</tr>
<tr>
<td>MR27</td>
<td>MTCE</td>
<td>8.6</td>
<td>10</td>
<td>&gt;0.16</td>
<td>0.017</td>
</tr>
<tr>
<td>MR17</td>
<td>MTCE</td>
<td>8.3</td>
<td>10</td>
<td>0.01</td>
<td>0.0051</td>
</tr>
<tr>
<td>MR27</td>
<td>MTCE</td>
<td>9.9</td>
<td>16</td>
<td>0.0036</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* CF, chick fibroblast; MTCE, Maitland-type chick embryo.

* Mice were inoculated with 0.25-ml amounts of vaccine at 7-day intervals.

* Partially purified.

In addition, smaller ED_{50} values were obtained in the guinea pig than in the mouse. The data indicate that irradiation is a satisfactory tool for the preparation of potent VEE virus vaccines.

Effect of irradiation on antigenicity. Table 3 lists the ED_{50} values for vaccines that were exposed to various doses of irradiation. A dosage of 10 x 10^6 r caused approximately a 100-fold decrease in the potency of the WI-38 vaccine, but had no deleterious effect on the MTCE vaccine. Exposure of the latter to an irradiation dose of 16 x 10^6 r caused approximately a 10-fold decrease in potency; no potency was demonstrable after a dosage of 32 x 10^6 r.

Resistance of vaccinated guinea pigs to challenge. Because guinea pigs immunized with irradiated VEE vaccines resisted challenge with 10^4.4 MLD_{50} of virulent VEE virus, it was of interest to determine the upper limits of protection afforded by vaccination. A total of 105 animals were immunized with three inoculations of 0.25-ml amounts of undiluted vaccine (MTCE) exposed to 8 x 10^6 r. The animals were divided into groups of 15 each and challenged 21 days later with graded doses of VEE virus ranging from 10^4.4 to 10^8.4 MLD_{50}. All vaccinated animals survived challenge. Serum samples were taken from two animals selected at random from each group immediately prior to challenge and 14 days postchallenge. Prechallenge sera contained high levels of SN antibody (5.1 to 7.3) and moderate to high levels of HI antibody (160 to >10,240). Five of six guinea pigs challenged with doses of 10^4.4 MLD_{50} or greater responded with a fourfold or greater increase in postchallenge HI titer.

Antibody response of rabbits to irradiated VEE vaccine. Table 4 shows the HI and SN antibody titers obtained in the sera of two rabbits given a

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Cell culture prep</th>
<th>ED_{50} (ml) per irradiation dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR25</td>
<td>MTCE</td>
<td>0.0036</td>
</tr>
<tr>
<td>MR27</td>
<td>MTCE</td>
<td>0.0046</td>
</tr>
<tr>
<td>50/2</td>
<td>WI-38</td>
<td>0.02</td>
</tr>
<tr>
<td>58/2</td>
<td>WI-38</td>
<td>0.00067</td>
</tr>
<tr>
<td>59/2a</td>
<td>9.7</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

* Animals vaccinated with two inoculations of 0.2 ml.

* Animals vaccinated with three inoculations of 0.2 ml.

* Maitland-type chick embryo.

TABLE 4. Hemagglutination-inhibiting (HI) and serum-neutralizing (SN) antibody response in rabbits vaccinated with irradiation-inactivated Venezuelan equine encephalitis vaccine

<table>
<thead>
<tr>
<th>Weeks postvaccination</th>
<th>Rabbit 1</th>
<th>Rabbit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reciprocal of HI</td>
<td>Log_{10} SN</td>
</tr>
<tr>
<td>1</td>
<td>5,120</td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>640</td>
<td>8.4</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
<td>ND</td>
</tr>
<tr>
<td>40</td>
<td>80</td>
<td>3.6</td>
</tr>
<tr>
<td>60 (booster)</td>
<td>20</td>
<td>3.3</td>
</tr>
<tr>
<td>61</td>
<td>5,120</td>
<td>8.4</td>
</tr>
<tr>
<td>62</td>
<td>&gt;20,480</td>
<td>8.4</td>
</tr>
<tr>
<td>63</td>
<td>10,240</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* ND, not done.
series of five injections of 0.5 ml of irradiated vaccine, lot MR27, on days 0, 3, 5, 9, and 11, followed by a booster dose of 0.5 ml at 15 months. High HI and SN titers occurred 1 week after completion of the initial vaccination series. The HI titer in one rabbit was still at a significant level 10 months later, but was barely detectable in the other rabbit. Both rabbits exhibited lower but significant SN titers at that time. A single booster dose of vaccine stimulated a rapid and substantial increase in HI and SN antibodies at least equal to the original peak titer levels.

**DISCUSSION**

VEE vaccines inactivated by gamma irradiation of virus suspensions prepared in tissue cultures exhibit high potencies. Irradiated vaccines stimulate the production of high levels of HI and SN antibodies when administered to animals in a series of two or more injections.

The potencies of irradiated vaccines prepared from infected WI-38 monolayer or MTCE tissue cultures differed little. However, the antigenicity of the WI-38 vaccine was more sensitive than that of the MTCE vaccine to the deleterious action of high doses (10 \times 10^4 \text{ r}) of radiation. This may be due to the presence of lower amounts of proteinaceous material in the WI-38 preparation. Polley (10) reported that influenza virus hemagglutinin of purified suspensions was destroyed more rapidly than infectivity, and that the addition of radioprotective agents such as histidine, ascorbic acid, or cysteine reversed this effect. Nagle medium (9), used for growth and maintenance of MTCE, contains greater amounts of radioprotective substances than the Basal medium Eagle used for WI-38 cells.

Of 6 guinea pigs challenged with doses of $10^{3.4} \text{ MICLD}_{50}$ or greater, 5 responded with a significant rise in HI antibody titer. Increased antibody production could be attributed to: (i) inability of the vaccine to produce a level of immunity sufficient to prevent subclinical infection by high challenge doses, or (ii) an anamnestic response to the large quantity of antigen in the challenge doses. The high SN antibody levels present at the time of challenge appear to support the latter concept.

An excellent immune response was obtained in rabbits. Although antibody levels decreased considerably 10 to 15 months after vaccination, significant levels of SN antibody were still present at that time, and a single booster dose rapidly restored circulating antibody to the original levels.

The data presented here indicate that inactivation by ionizing radiation may be an excellent method for the inactivation of virus in the preparation of virus vaccines. This conclusion is supported by the degree of protection afforded vaccinated guinea pigs against challenge with doses up to $10^{3.4} \text{ MICLD}_{50}$ of virus.

Highly potent inactivated VEE vaccines prepared by irradiation with gamma rays may be applicable to immunization of animals, such as equines and fowls, in an attempt to break the VEE virus-arthropod cycle of infection in epidemic and endemic areas. An irradiated vaccinia virus vaccine has been tested in humans previously immunized against smallpox with a live vaccine (3). Administration of the irradiated (noninfectious) antigen stimulated an antibody response and produced a maculopapular or vesicular reaction of the delayed hypersensitivity type.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

15. Reitman, M. 1969. Nonviable Venezuelan equine encephalo-