Immune Response in Primates Vaccinated with Duck Embryo Cell Culture Rabies Vaccine

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Adult rhesus monkeys (Macaca mulata) were vaccinated with four inactivated rabies vaccines, including two cell culture vaccines, one zonal purified cell culture vaccine, and a 10% extracted duck embryo vaccine. The vaccines were potency tested by both National Institutes of Health (NIH) and Habel methods and passed one or both tests. However, a vaccine having acceptable potency by one method frequently failed or was marginal by the other procedure. Groups of three monkeys were inoculated with each vaccine by one of two schedules. The first consisted of four weekly 1-ml doses followed by a 1-ml booster dose at 6 months, and the second consisted of seven daily 1-ml doses of vaccine with no booster. Both zonal purified and extracted duck embryo vaccines induced detectable neutralizing antibody by day 7 with either schedule, and antibody titers elicited by the cell culture vaccine remained high through 210 days. However, antibody titers produced by the 10% duck embryo vaccine dropped sharply after their 28-day peak. Duck embryo cell culture vaccines with low or marginal potency as measured by Habel or NIH tests still produced rapid, high levels of serum-neutralizing antibody in primates. LD_{50} or NIH and Habel tests as measured in mice were not necessarily good indices of antibody response in the primate host. The need for a cell culture potency test that will yield a more predictable correlation with the definitive host’s antibody response is discussed.

Several studies of antibody response in primates vaccinated with rabies vaccine have been reported (5, 7). Wiktor and Koprowski successfully immunized rhesus monkeys with a live high egg passage rabies vaccine propagated in WI-38 cells (7). Sikes et al. (5) showed that highly purified inactivated cell culture rabies vaccine would protect monkeys from challenge with rabies street virus.

In a recent study (3), we described the preparation and purification of rabies virus vaccines grown in duck embryo cell culture. These vaccines were of high purity, acceptable National Institutes of Health (NIH) potency, and satisfactory immunogenicity as measured by sero-conversion in rabbits receiving one dose of vaccine. In the present study, four vaccines—two of the cell culture vaccines, one purified cell culture vaccine, and one 10% extracted duck embryo vaccine—were compared for their capacity to produce rabies serum-neutralizing (SN) antibodies in rhesus monkeys (Macaca mulata). Groups of three adult rhesus monkeys were vaccinated intramuscularly with each vaccine. Two vaccination schedules were studied, one simulating preexposure and the other postexposure immunization.

The results of both vaccination schedules show that rabies duck embryo cell culture vaccine will produce a rapid, sustained SN antibody response in primates.

MATERIALS AND METHODS

Virus. The CVS strain of fixed rabies virus was adapted to duck embryo cell culture as described in a previous publication (3). This virus seed was used in the production of all cell culture vaccines.

The duck embryo extracted vaccine was prepared from CVS fixed rabies virus passed in mouse brain. This vaccine is an inactivated 10% extract of rabies-infected duck embryos (4).

Media. All cell culture vaccines were prepared with F-10 or 199 medium supplemented with 0.5% human albumin. The duck embryo cell culture monolayers were grown in 64-oz (ca. 1,900-ml) Owens-Illinois bottles containing 125 ml of medium. The average lot of vaccine prior to purification was 30 liters.

Zonal centrifugation and purification. The cell culture vaccine was concentrated and purified in
a sucrose density gradient with a K-VI rotor driven in a K-II B experimental zonal centrifuge. The K-VI rotor was obtained through the courtesy of N. G. Anderson, Molecular Anatomy Program, Oak Ridge National Laboratories, Oak Ridge, Tenn. (1). Up to 30 liters of vaccine was concentrated to 400 to 800 ml in the 20 to 50% fractions of sucrose. These sucrose fractions were pooled and reconstituted to one-tenth of the original 30-liter starting volume (10x vaccine now containing 5% sucrose) with medium 199 containing 0.5% human albumin; they were then inactivated with β-propiolactone (BPL). A detailed description of the purification by rate zonal centrifugation is included in a recent publication (3).

**Antibiotics and preservatives.** All cell culture vaccines contained 50 μg of neomycin sulfate/ml and 1:10,000 Merthiolate (Thimerosal, Eli Lilly & Co.). The extracted duck embryo vaccine also had 1:10,000 Merthiolate as a preservative.

**Virus inactivation and lyophilization.** The cell culture vaccines were inactivated while stirring, with 1:8,000 BPL for 2 h at room temperature, followed by 72 h at 4 °C. By colorimetric assay, residual BPL could not be detected in the vaccine after 72 h.

The extracted rabies duck embryo vaccine was inactivated while stirring with 1:4,000 BPL at 4 °C for 48 h.

All vaccines were lyophilized in a Stokes orVirTis dryer for 72 to 96 h. The cell culture vaccines contained a final concentration of 3% NZ-Amine-AS (Sheffield Laboratories) as bulking agent. The 10% extracted lot of rabies vaccine was lyophilized with a 5% suspension of lactose-gelatin as bulking agent. Each dried lot of vaccine was tested for moisture (P₂O₅ method), sterility (thioglycolate broth at 25 and 36 °C), and lack of infectivity (intracerebral inoculation of undiluted reconstituted vaccine into twenty 12- to 14-g mice). Only vaccines with low moisture content (<3%) which passed the sterility tests and showed no infectivity were used for animal vaccination.

**Adjuvant.** All lyophilized cell culture vaccines were reconstituted with sterile pyrogen-free water containing 1 mg of AlPO₄/ml as adjuvant. The 10% duck embryo extracted vaccine was reconstituted with sterile pyrogen-free water.

**LD₅₀ test.** The standard LD₅₀ test was used for virus titration. Tenfold dilutions of vaccine were inoculated intracerebrally into 12- to 14-g mice. Mice were held for 14 days, and only mice dying after the 5th day were used in calculating the LD₅₀. The Reed-Muench method was used for all calculations and titer was expressed as the amount of virus contained in 0.05 ml of vaccine (8).

**Potency test.** Vaccines were assayed for potency by both the standard NIH and Habel tests. A potency ratio of 0.30 is necessary to pass the NIH test, and at least 1,000 LD₅₀ of protection are necessary to pass the Habel test. All vaccines were tested as being equivalent to a 10% suspension of brain tissue (by the NIH test) even though the cell culture vaccines contained only about one-hundredth the amount of protein nitrogen of the NIH standard or the 10% extracted duck embryo vaccine. For the Habel test, the cell culture vaccines were diluted 1:10 and the extracted vaccine was diluted to a 0.5% suspension. The NIH reference (176 or 178) was used as a control with each Habel test. A complete description of NIH and Habel test procedures is included in Laboratory Techniques in Rabies (8) and in a previous publication (3).

**Immunogenicity test in primates.** Twenty-eight individually housed young adult rhesus monkeys (Macaca mulata) weighing 3.5 to 4 kg were selected for vaccination with the four rabies vaccines. Groups of three monkeys were inoculated with each vaccine. Groups A, B, C, and D each received a total of five 1-ml intramuscular doses of vaccine consisting of four weekly doses and one booster dose 6 months after the initial vaccination. Groups E, F, G, and H each received seven daily intramuscular doses of vaccine with no booster dose. These two schedules were designed to simulate both pre- and postexposure immunization.

A prevaccination blood sample and additional blood samples were collected from each monkey at 7, 14, 28, 60, 120, and 210 days after initial immunization. The sera from each sample were inactivated at 56 °C for 30 min. Each serum sample was titrated for SN antibody by the standard SN test of intracerebral inoculation of adult mice with 10-fold dilutions of incubated sera containing at least 100 LD₅₀ of fixed rabies virus (8). The SN titers (50% end points) were calculated by the Karber method and represent the reciprocal of the highest serum dilution showing neutralization.

**RESULTS**

The potency test results for the four vaccines used in this study are tabulated in Table 1. Each vaccine was tested for potency by both NIH and Habel methods shortly before the initiation of the experiment. In addition, the two cell culture vaccines (A and B) each had been tested earlier by the Habel method. These two cell culture vaccines were 19 to 21 months old at the time of initiation of primate vaccination. Neither lot of vaccine had acceptable potency by the NIH test. Although both vaccines A and B had been produced by the same methods, vaccine A (T-64722-B) had maintained a high level of protection by the Habel test, whereas vaccine B (T-64957-B) remained rather marginal.

The zonal purified vaccine was 14 months old at the time of primate vaccination and had acceptable NIH and Habel potency, but the level of protection by the Habel method was marginal.

The 10% extracted lot of duck embryo vaccine was 6 months old when used in this study and had acceptable potency by both NIH and Habel methods.
All vaccines were tested (by the NIH method) as being equivalent to a 10% suspension of brain tissue. The three cell culture vaccines, in fact, contained about one-hundredth the amount of protein nitrogen of a brain tissue vaccine or the extracted duck embryo vaccine.

These vaccines were selected for this study because of their wide differences in potency and age at the time of primate vaccination. If potency, as currently measured, and stability are reasonably good indices of antigenicity, this should be reflected in antibody response in the vaccinated monkeys. The results of this study do not necessarily support this theory.

**Antibody response.** The individual antibody response of each rhesus monkey over the 210-day test period is given in Table 2. Table 3 shows the average SN titers for each group.

Looking first at groups A through D (four weekly vaccine doses and a booster at 6 months, Table 3), we can conclude that cell culture vaccines A and B first showed detectable antibody response at 14 days, which then peaked for group A at 28 days and continued rising for group B through the entire test period. Both groups A and B showed a typical

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**Table 1. Rabies duck embryo vaccine potency testing**

| Lot no.                  | LD<sub>50</sub> (log 10/0.03 ml of vaccine postlabeling) | Age of vaccine (mo.) | NIH ratio<sup>a</sup> | Habel index<sup>b</sup> | Test |
|--------------------------|----------------------------------------------------------|----------------------|------------------------|-------------------------|------|
| T-64722-B, cell culture  | 4.1                                                      | 9                    | >378,000 A             |                         |      |
| 17                       | 17                                                       | 21                   | >248,000 B             |                         |      |
| 21                       | 15                                                       | 0.21                 | 188,000 C              |                         |      |
| T-64957-B, cell culture  | 5.1                                                      | 5                    | 48,000 A               |                         |      |
| 15                       | 19                                                       | 0.22                 | 25,000 B               |                         |      |
| T-65148-D, zonal purified cell culture | 4.5 | 14 | 0.42 | 4,300 C | C |
| T-66494, 10% extracted duck embryo | 6 | 0.36 | 27,000 C | C |

<sup>a</sup> NIH ratio of at least 0.30 necessary to pass potency.

<sup>b</sup> At least 1,000 LD<sub>50</sub> of protection necessary to pass Habel test.

* NIH reference vaccines used as controls with each Habel test.

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**Table 2. Rabies serum neutralization (SN) titers in primates**

| Monkey<sup>a</sup> | SN titers at Pre-bleed | 7 days | 14 days | 28 days | 60 days | 120 days | 210 days |
|---------------------|------------------------|--------|---------|---------|---------|----------|----------|
| 5075-A              | <4                     | <4     | <4      | 64      | 25      | 10       | >128     |
| 5076-A              | <4                     | <4     | 16      | >90     | 40      | 11       | >128     |
| 5077-A              | 5                      | 4      | 80      | 36      | 13      | >128     |
| 5078-B              | <4                     | <4     | 4       | 63      | 40      | <16      | >159     |
| 5079-B              | <4                     | <4     | 25      | 80      | 281     | 445      | >1,262   |
| 5080-B              | <4                     | <4     | 32      | 56      | 256     | 448      | >1,024   |
| 5081-C              | <4                     | <4     | 7       | >90     | <64     | 45       | 470      |
| 5082-C              | 4                      | 9      | 123     | >357    | 711     | 1,122    | >1,024   |
| 5083-C              | <4                     | 10     | 70      | >357    | 283     | 100      | >512     |
| 5084-D              | <4                     | 10     | 16      | 56      | 14      | 21       | >128     |
| 5085-D              | <4                     | 9      | 9       | 126     | 45      | 28       | >128     |
| 5086-D              | <4                     | 4      | 5       | 126     | 63      | 50       | >186     |
| 5087-E              | <4                     | 4      | 56      | 79      | 113     | 177      | 256      |
| 5088-E              | <4                     | <4     | 32      | 56      | 64      | 68       | 256      |
| 5089-E              | <4                     | 4      | 141     | 159     | 282     | 356      | >317     |
| 5090-F              | <4                     | 5      | 142     | 317     | 500     | 98       | >282     |
| 5091-F              | <4                     | <4     | 80      | 317     | 504     | 354      | >512     |
| 5092-F              | <4                     | 4      | 159     | >357    | 224     | 340      | >512     |
| 5093-G              | 4                      | 71     | 141     | >357    | 113     | >256     | >619     |
| 5094-G              | <4                     | 71     | 109     | 157     | 319     | 201      | >500     |
| 5095-G              | <4                     | 27     | 99      | 112     | 40      | 16       | 49       |
| 5096-H              | <4                     | 16     | 90      | 32      | 14      | 10       | >56      |
| 5097-H              | <4                     | 28     | 80      | 99      | 25      | 14       | 55       |
| 5098-H              | <4                     | 25     | 32      | 123     | 28      | >64      | >398     |

<sup>a</sup> Groups A-D, monkeys 5075-5086, received four weekly 1-ml doses and one booster dose at 180 days.

Groups E-H, monkeys 5087-5098, received seven daily 1-ml doses of vaccine.
Table 3. Immunogenicity of rabies vaccine in primates

| Group<sup>a</sup> | Type of vaccine | Avg SN titer<sup>b</sup> at day | 0 | 7 | 14 | 28 | 60 | 120 | 210 |
|------------------|-----------------|---------------------------------|---|---|----|----|----|-----|-----|
| A                | Cell culture A  | <4                             | <4| <4| 8  | 78 | 34 | 11  | 128 |
| B                | Cell culture B  | <4                             | <4| <4| 20 | 66 | 192| 300 | >815|
| C                | Zonal purified  | 817                           | 67 | ≥270| 497| 642 | ≥699|
| D                | 10% extracted   | <4                             | 7 | 10 | 103| 41 | 33  | ≥144|
| E                | Cell culture A  | <4                             | <4| <4| 76 | 98 | 153| 200 | ≥276|
| F                | Cell culture B  | 44                             | 127| <330| 409| 294| ≥389|
| G                | Zonal purified  | <4                             | 55 | 116| ≥209| 09 | 157| ≥158| ≥399|
| H                | 10% extracted   | <4                             | 23 | 68 | 85 | 22 | ≥29 | 170 |

<sup>a</sup> Each group consisted of three monkeys. Groups A through D were vaccinated with one dose of vaccine at 0, 7, 14, and 28 days, and with a booster dose at 6 months. Groups E through H were vaccinated with one dose of vaccine on days 0, 1, 2, 3, 4, 5, and 6, and received no booster dose.

<sup>b</sup> Reciprocal of serum dilution, determined by the Karber method.

The anamnestic response 30 days after the booster dose. Group C, the zonal purified cell culture vaccine, exhibited detectable SN antibody at 7 days, which peaked at 60 days, leveled off slightly at 120 days, and then jumped up again 30 days after the booster dose.

Group D (10% duck embryonic vaccine) also showed detectable antibody at 7 days, which peaked at 28 days, dropped rather sharply over the next 92 days, and again rose sharply 30 days after the booster dose.

Group C, the zonal purified vaccine, was superior to the other three vaccines in terms of level and duration of immunity over the entire 210-day test period.

Looking next at groups E through H (seven daily doses of vaccine with no booster), we see group E (cell culture vaccine A) showing detectable antibody at 14 days, which continued to rise over the next 6 months. Group F (cell culture vaccine B) exhibited detectable antibody at 7 days, and antibody climbed sharply through 60 days and remained at a high level through 210 days.

Group G (zonal purified vaccine) showed the highest level of antibody of all groups at 7 days, which continued rising and maintained a high titer through the 210-day test period.

Group H (10% duck embryonic vaccine) also showed SN antibodies at 7 days, which rose to a high at 28 days but never achieved the antibody levels of the other three cell culture vaccines.

**DISCUSSION**

The results of this study indicate that duck embryo cell culture vaccines with AlPO<sub>4</sub> adjuvant (as illustrated by T64722B and T64957B) stored for almost 2 years at 4°C, with low NIH or Habel potency (or both for lot T64957B), have the capacity to stimulate high, sustained SN antibody levels in primates. Both of the cell culture vaccines effectively induced prolonged antibody responses by either vaccination schedule. Also, the low-potency cell culture vaccine yielded high titers, especially on the seven-dose schedule.

The 10% extracted duck embryo vaccine showed measurable antibody at 7 days postvaccination by the five-dose booster schedule, but antibody titers were generally lower than those seen with cell culture vaccine B or the zonal purified vaccine. The SN antibody levels elicited by extracted duck embryo vaccine reached a peak at 28 days and then dropped sharply through 120 days prior to the booster dose.

With the seven-dose schedule, the antibody level produced by the 10% extracted duck embryo vaccine was lower at 7 days than that produced by the zonal purified vaccine and never approached the titers obtained with the other three cell culture vaccines after 14 days.

The zonal purified vaccine is notable for its rapid induction and sustained high levels of antibody by either vaccination schedule. Despite the fact that resistance to infection may be more important at the cellular level than the humoral, until a better measure of protection is found, rapidity of appearance and level of circulating SN antibodies must be considered of major importance in selecting a vaccine for postexposure immunity (6).

This study again emphasizes that the SN antibody response in primates or other mammals may not be entirely predictable from the NIH or Habel potency test results (3). The repeated testing of a vaccine lot by either method may give extremely variable results. Also, the mouse LD<sub>50</sub> test is not an entirely reliable index of antigenicity. For many years now, it has been obvious to us after producing hundreds of lots of production duck embryo vaccine that LD<sub>50</sub> is a crude measure of expected potency. In fact, to my knowledge, a definitive study of the relationship of NIH potency ratio and SN response in man has not been done; i.e., a vaccine with a relatively low NIH ratio (0.20 to 0.30) may in fact be as antigenic in man as a vaccine with an NIH ratio of say 1.0.
Since speed of induction and level of antibody response may be critical for survival in postexposure immunity, the 10× concentrated zonal purified vaccine would obviously be the vaccine of choice of the four studied. For that matter, a 100× or greater concentrated vaccine (based on a recent primate study; 5) might be ideal. However, one must consider the realities of test and production costs in determining a human product. In my experience, a 10× vaccine is feasible in terms of vaccine yield, production, and ultimate cost of product. By zonal centrifugation, one can easily produce a vaccine of 100× or 200× concentration which would result in enormous antigenic values and virus titers. However, the yield would be small and starting material would be measured in thousands of liters, which is prohibitive in terms of production and cost.

Also, the experimental data reported here may be an indication that the 10× duck embryo cell culture vaccine could be sufficient for pre- or postexposure immunity if SN titer is a reliable index of protection. The real problem seems to be finding an adequate method to measure predictably the expected potency in a definitive host. This ultimately may require a potency test far different from those on which we currently rely.

Several years ago, Cabasso (2) faced this problem when he was required to potency test veterinary rabies vaccine in the guinea pig. Many lots of vaccine failing the potency test were antigenic and protected the definitive host, the dog, from death when challenged with street virus. Veterinary rabies vaccines are still required to pass the guinea pig test prior to use in the dog. We appear to be facing a similar problem today in human rabies vaccine potency. We have the means of producing antigenic cell culture vaccines with a very low content of foreign protein; however, we still use the mouse to measure potency. Recent data suggest that the antibody response in a host more closely related to man, such as the primate, may be a better index of human response and protection.

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