Zonal-Centrifuged Purified Duck Embryo Cell Culture Rabies Vaccine for Human Vaccination

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Rabies virus produced in duck embryo cell culture was concentrated from volumes of 14 to 30 liters to 400 to 800 ml by zonal centrifugation. Virus titers of peak fractions were from 100- to 1,000-fold greater than those of the starting material. Vaccines were prepared by combining fractions with peak virus titers and diluting back to 10 times concentration. The resulting \( \beta \)-propiolactone-inactivated vaccines, when prepared as lyophilized vaccines with AIPO\(_4\) adjuvant diluents, were low in protein nitrogen (0.01 mg/ml), and three of four lots passed the National Institutes of Health potency test when tested as equivalent to a standard 10\% suspension of duck embryo or mouse brain tissue vaccine. These vaccines also induced good sero-conversion in adult rabbits after a single 1-ml dose of vaccine. Guinea pigs sensitized with zonal-centrifuged purified duck embryo vaccine (with AIPO\(_4\) adjuvant) did not exhibit anaphylactic shock reactions when challenged with homologous vaccine. Also, no anaphylactic shock reactions were observed when guinea pigs were sensitized with either a 10\% experimental duck embryo vaccine or cell culture vaccine and then challenged with the zonal-purified vaccine. However, guinea pigs sensitized with cell culture or zonal-purified vaccine and then challenged with the 10\% experimental vaccine did show slight transitory congestion. The 10\% experimental whole duck embryo vaccine was responsible for all observed anaphylactic shock reactions whether homologous or heterologous.

The initiation of the Pasteur treatment (10) ushered in a new era in human vaccine prophylaxis. The generally long incubation period associated with human rabies infection made possible the effective treatment of this viral disease after exposure. However, the vaccine was very crude, containing high concentrations of neural tissue, lipid, and protein. Therefore, local and systemic reactions, as well as neuroparalytic accidents, were not uncommon.

Other improved vaccines followed, including the partially inactivated sheep brain vaccine of Fermi (4) and the phenol-inactivated rabbit brain vaccine of Semple (12). Since both of these vaccines were of neural tissue origin, post-vaccinal neuroparalytic involvements were still significant.

The chick embryo origin (CEO) vaccines, derived from both low and high egg passages (LEP and HEP), of the Flury rabies strain were significant improvements over the neural tissue products because of their virtual freedom from the encephalitogenic factor (8, 9). These vaccines have had wide acceptance in the veterinary field. However, they still have the disadvantages of high extraneous protein content and the occasional induction of local and systemic reactions, as well as anaphylactic shock. Whereas the LEP vaccine had certain inherent dangers for human vaccination, the HEP failed to live up to expectations because of its failure to multiply in man (13). More recently, the inactivated duck embryo vaccine has successfully replaced the neural tissue vaccines for human vaccination in this country, virtually eliminating the encephalitogenic factor and hence neuroparalytic involvement (11). However, this vaccine also contains extraneous protein and lipid, and some local and systemic reactions continue to occur, especially in subjects allergic to egg proteins.

Most recently, vaccines have been prepared from rabies virus grown in chicken, duck, hamster, pig, monkey, rabbit, mouse, dog, and human diploid cells. Although these vaccines have had the advantages of high purity and freedom from the encephalitogenic factor, they generally lack the virus titers and immunogenicity of neural tissue vaccines. Therefore, potency of the products is frequently marginal as indicated by the conventional testing methods (1, 3, 5-7,
13–15). Wiktor et al. (16) prepared a purified cell culture vaccine from rabies virus seed grown in BHK-21 cells. The virus was purified by a combination of zinc acetate precipitation, Sephadex chromatography, high-speed centrifugation, and forced dialysis. The development by Anderson et al. (2) of large-capacity zonal centrifuges made possible the concentration and purification of large volumes of virus fluid.

In this report, the results of experiments in which rabies vaccine produced in duck embryo cell culture is purified and concentrated by density gradient centrifugation are presented. This procedure resulted in a product of high purity, acceptable virus titers, and satisfactory immunogenicity as measured by the National Institutes of Health (NIH) test and by sero-conversion in adult rabbits.

MATERIALS AND METHODS

Virus. The CVS strain of fixed rabies virus was passed once in 7-day-old duck embryos. After an additional 14 days of incubation, the infected embryos were harvested and ground in a Waring Blender with a phosphate-buffered lactose diluent. This emulsion was centrifuged for 10 min at 1,000 rev/min, and the supernatant fluid was collected as a 10% suspension of rabies virus duck embryo seed. This seed was used to infect 7-day-old duck embryos which were harvested after 9 days of incubation and trypsinized by conventional methods; the infected cells were seeded in bottles as duck embryo cell cultures. After 3 to 5 days of incubation, the medium was harvested and dialyzed to five times concentration against Carbowax 6,000. This concentrated virus was used to inoculate young adult mice intracerebrally.

The brains of these animals were harvested when the animals were moribund (5 to 7 days). A 10% suspension of rabies-infected mouse brain was prepared by grinding the brains in a Waring Blender with sterile pyrogen-free water diluent. This emulsion was used to infect 7-day-old eggs. After an additional 7 days of incubation, the embryos were harvested, trypsinized, and planted as infected cells in 500F bottles. The medium from these bottles was harvested in 3 to 5 days, and again was inoculated intracerebrally into young adult mice. This method of alternately passing the virus seed in mouse brain and duck embryo cell culture was repeated five times in each system, followed by an additional seven to nine passages in duck embryo cell culture after the last passage in mouse brain. This resulted in a virus seed which consistently titrated between $10^{-4}$ and $10^{-4.4}$ MLD$_{50}$/0.03 ml in adult mice (Table 1).

Media. All cell culture passages were made with F-10 medium supplemented with 3% fetal calf serum or 0.5% human albumin in either 500F bottles with 30 ml of medium or Owens-Illinois bottles with 100 to 125 ml of medium.

Zonal centrifugation. For quantities of virus up to about 30 liters, the B-IX zonal centrifuge rotor was used. For larger quantities of virus, the K-VI rotor, driven in a K-11B experimental zonal ultracentrifuge, was used. (The K-VI rotor was obtained through the courtesy of N. G. Anderson, Anderson Molecular Anatomy Program, Oak Ridge National Laboratory, Oak Ridge, Tenn.) Both rotors operate on the principle of continuous-flow isopycnic banding. The K-VI rotor is basically the K-II rotor with the addition of a coaxial flow chamber which removes large particulates from the fluid flowing into the centrifuge prior to its passage across the gradient. The reorienting gradient technique was used with the K-VI rotor; i.e., the rotor is loaded and unloaded at rest. Both dynamic unloading and the reorienting gradient technique were used with the B-IX rotor. Our experience has been that the reorienting gradient technique results in better resolution of the materials banded in the gradient. Stepwise, diffusion gradients were used in all experiments and were produced by pumping the appropriate volumes of buffered sucrose into the rotor. For the K-VI rotor, the gradient consisted of 1,600 ml of 1.8% buffered sucrose, 1,000 ml of 30% buffered sucrose, and 1,000 ml of 60% buffered sucrose. In early experiments with the B-IX rotor, the gradient consisted of 400 ml of 3% and 350 ml of 60% buffered sucrose. Better separation of the virus and contaminating extraneous materials was obtained when the gradient consisted of 300 ml of 2%, 100 ml of 30%, 140 ml of 40%, and 200 ml of 60% buffered sucrose. This gradient was used for the majority of the experiments.

Gradient shape. Gradient shape was determined by measuring the refractive index (RI) of each fraction with a Bausch and Lomb Abbé refractometer.

Ultraviolet adsorption spectra. Absorbancy between 250 and 350 nm was determined for all fractions by use of a Cary 15 recording spectrophotometer.

Virus inactivation. All purified rabies cell culture vaccines were inactivated by stirring with 1:8,000 β-propiolactone (BPL) for 2 hr at room temperature, followed by 72 hr at 4 C. By colorimetric assay, residual BPL could not be detected in the vaccine after 72 hr.

Preparation of purified vaccines. Selected high virus titer fractions were pooled and diluted with sterile pyrogen-free water or Hanks basic salt solution to a final sucrose concentration of 5 to 8%. One lot of vaccine was dialyzed against running tap water to 10% sucrose (T65148D). After 48 hr of BPL inactiva-

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Table 1. Rabies cell culture vaccine consistency

| Lot no. | LD$_{50}$, log$_{10}$ |
|---------|---------------------|
| T-61639 | 4.3                 |
| T-61646 | 4.4                 |
| T-61655 | 4.2                 |
| T-61660 | 3.8                 |
| T-61666 | 4.4                 |
| T-61678 | 4.3                 |
| T-61681 | 4.5                 |
| T-61709 | 4.4                 |
| T-61732 | 4.4                 |
| T-61777 | 4.1                 |
tion, the pH of each vaccine was adjusted to 7.3 to 7.4 when necessary. Both one- and five-dose sample lots were filled and lyophilized (in a Stokes or VirTis dryer for 72 to 96 hr). Each dried lot of vaccine was tested for moisture (P₂O₅ method), sterility (thioglycolate broth at 25 and 37 C for 7 days), and innocuity (intracerebral inoculation of undiluted reconstituted virus into twenty 12- to 14-g mice). Only vaccine lots passing the sterility and innocuity tests were used for potency testing.

**Adjuvant.** Both the standard cell culture and zonal-purified liquid vaccines were supplemented with 1 mg of AlPO₄₅ per ml as an adjuvant. All lyophilized vaccines were reconstituted with sterile pyrogen-free water containing 1 mg of AlPO₄₅ per ml as adjuvant.

**Potency testing.** All purified cell culture vaccines were assayed for potency by the standard National Institutes of Health (NIH) test. All vaccines were tested as being equal in protein concentration to a standard 10⁻⁶ brain suspension vaccine, even though the purified vaccines contained only about one-hundredth the amount of protein nitrogen of a 10⁻⁶ brain suspension or standard NIH reference vaccine.

**LD₅₀ tests.** Density gradient fractions, cell culture rabies vaccines, and purified cell culture rabies vaccines were tested for virus content by the intracerebral inoculation of 12- to 14-g mice with 10-fold dilutions of virus sample. The LD₅₀ was calculated by the Reed-Muench method and was expressed as the amount of virus contained in 0.03 ml of vaccine. All lyophilized samples were reconstituted with sterile, pyrogen-free water prior to testing.

**Immunogenicity tests in rabbits.** Groups of three rabbits were vaccinated with either a standard duck embryo cell culture vaccine (both liquid and lyophilized) or a zonal purified duck embryo cell culture vaccine (both liquid or lyophilized). Each rabbit was given one human dose of vaccine (1 ml). Blood samples were collected from each rabbit at 0, 5, 10, 14, 28, and 60 days. The serum was collected from each blood specimen and inactivated at 56 C for 30 min. The sera were pooled by groups and titrated for rabies antibodies by the standard serum neutralization (SN) test of intracerebral inoculation of adult mice with twofold dilutions of incubated sera each containing 22 LD₅₀ of fixed rabies virus. SN titers represent the reciprocal of the highest dilution showing neutralization.

**Anaphylactic shock.** Three rabies vaccines were tested for anaphylactogenic activity in guinea pigs: (i) 10⁻⁶ experimental whole duck embryo vaccine, (ii) duck embryo cell culture vaccine with AlPO₄₅ adjuvant, (iii) zonal purified duck embryo vaccine with AlPO₄₅ adjuvant. A 0.1-ml dose of each vaccine was inoculated intradermally in the pectoral region of four 400- to 500-g guinea pigs. Some 26 to 30 days later, the guinea pigs in each group (along with uninoculated controls) were challenged intravenously with 1 ml of the homologous or a heterologous rabies vaccine. Each guinea pig was individually observed and scored from negative to plus 4 on the basis of 11 typical symptoms of anaphylactic shock.

**RESULTS**

**Zonal centrifugation.** Much of the virus used in early experiments had been frozen in large volumes at the time of harvest. Upon thawing, masses of aggregated protein formed, which on several occasions clogged the B-IX rotor. Various filtration techniques were tried to remove this material with little success, mainly because of rapid clogging of the filters and significant loss of virus. This thawed fluid could be centrifuged with the K-VI rotor without detectable loss of infectious virus. The aggregated masses were collected in the coaxial trap, and essentially no particulate contamination of the gradient occurred. When freshly harvested culture fluid was processed through the B-IX rotor, no problems were encountered with clogging. The majority of experiments with the four-step gradient were done with freshly harvested virus. However, there is little evidence that the freezing and thawing per se significantly affected viral antigenicity.

Figure 1 shows the results of a typical experiment with the K-VI rotor. The rate of flow through the rotor was 9.0 liters/hr, which resulted in removal of all detectable virus. The virus was sharply banded in a region of the gradient well removed from the majority of the contaminating substance found in the culture fluid. Figure 2 is an electron micrograph of virus from the peak region of the gradient.

Figure 3 shows the results of a typical experiment in which the B-IX rotor was used to concentrate and purify virus which had been stored frozen, but had been successfully filtered through Millipore prefilters and SM filters before centrifugation. The two-step gradient was used in this centrifugation, and the separation of the virus from the extraneous material in the gradient was not entirely satisfactory. The use of the three-step gradient was considered in an effort to increase the separation of the extraneous material and virus in the gradient.

Figures 4 and 5 show the results of centrifugation of two separate harvests from the same cell cultures. The first harvest was from cells grown on media supplemented with calf serum, and the second harvest was from cells grown with 0.5% human albumin-supplemented medium. A marked difference in the sedimentation pattern of the particulates from the two harvests was observed. Figure 4 indicates that the majority of the extraneous material is found in a region of the gradient lighter than the isopycnic density of the virus, and Fig. 5 indicates that most of the extraneous material banded in the gradient in a region heavier than the virus. In both cases, the virus was found in the same region of the gra-
dient. However, marked differences were noted in the potency of the vaccine produced from the two concentrations (see Table 4, T65148 and T65149).

The results tabulated in Table 2 indicate that two lots of cell culture vaccine with original virus titers of $10^{4.3}$ to $10^{4.4}$ logs were concentrated by zonal centrifugation to 100 times at the peak fractions (T-61639, T-61728-A and -B). The other two lots of vaccine (T65148 and T65149) exhibited a 1,000 times increase in virus titer at the peak fractions. Therefore, in all four experiments, between 14 and 30 liters of vaccine were banded in about 15 to 20 fractions ranging from 20 to 40 ml each, with all significant titratable virus contained in about 400 to 800 ml. The virus titer of the pooled vaccine fractions (prior to dilution) ranged from $10^{4.1}$ to $>10^6$ logs/0.03 ml and depended on the number of fractions included in the pool.

**Potency testing.** Unfortunately, no really specific standard test exists for potency testing cell culture rabies vaccines. Most workers in the past have used either the NIH test, the currently accepted test for rabies vaccines for human use, or the Habel test, formerly the standard test for human vaccines. Most workers prefer the Habel test, an immunity breakdown type of test in which all animals receive the same amount of vaccine and are then challenged with graded doses of virus. An acceptable vaccine must protect mice against at least a 1,000 LD$_{50}$ virus challenge. The NIH test is an antigen extinction test in which the mice receive graded doses of vaccine and are then challenged with a fixed amount of virus. A reference vaccine is run with the test vaccine, and

the ED$_{50}$ (effective dose) of the test vaccine is divided into the ED$_{50}$ of the reference vaccine to give a potency ratio. A ratio of 0.30 is necessary to pass the NIH test. The preference for the Habel test is primarily due to the fact that the NIH test was designed for testing vaccines with high protein content, and is therefore really not an ideal method for evaluation of a cell culture vaccine with low protein nitrogen.

Although the Habel test is not an ideal test for cell culture vaccines, we have found that vaccines which have very low potency when measured by the NIH test will frequently have very high potency indices by the Habel method, as illustrated in Table 3.
We, therefore, decided to subject our zonal-purified vaccines to the severest type of potency testing. Each vaccine was tested by the NIH method as being equal to a standard 10% suspension of brain tissue, whereas, in fact, each vaccine contained about one-hundredth the amount of protein nitrogen of either a standard duck embryo vaccine or the NIH reference. No extrapolation of ratio was made, based on the reduced amount of protein in the purified cell culture vaccines.

The potency test results indicate that lot T-61728-A, a liquid product, did not pass the NIH test; however, its lyophilized counterpart had an acceptable potency (Table 4). Lyophilized lots T-65148 and T-65148-D-dialyzed also had good potency, whereas lot T-65149 (the second harvest of T-65148 with a 0.5% human albumin supplement) did not pass the NIH test.

**Antibody response.** Rabbits were vaccinated with a single 1-ml dose of vaccine. Two lots of cell culture rabies vaccine were compared for their abilities to induce neutralizing antibodies in rabbits. One vaccine was a regular lot of cell culture rabies, lot T-61639, which failed the NIH test but passed the Habel test. The liquid portion of this vaccine induced a good, rapid SN response in rabbits, which reached a peak titer in 28 days (Fig. 6). However, the lyophilized fraction
showed no response at all for the first 14 days, and only a minimal SN titer thereafter (Fig. 6).

The other vaccines were prepared from zonal centrifuge peak virus titer fractions. The liquid purified vaccine also induced a good SN response which was maximal in 28 days (Fig. 6). The lyophilized portion of this vaccine had a somewhat long delay period prior to antibody induction (10 days), but then showed a gradual rise in SN titer, which was maximal in 60 days (Fig. 6). It is of interest that the liquid portion of the vaccine failed the NIH test, whereas the lyophilized fraction passed this same test.

Anaphylactic shock. The results of the anaphylaxis experiments are tabulated in Tables 5 and 6. All control guinea pigs were negative for symptoms of anaphylactic shock when challenged with any of the three duck embryo vaccines. However, all guinea pigs sensitized with and then later challenged with extract of whole duck embryo vaccine died of severe anaphylactic shock within 3 min of challenge.

Also, guinea pigs sensitized with cell culture or zonal-purified rabies vaccines and then challenged with whole duck embryo vaccine showed slight transitory congestion. However, no anaphylactic shock reactions were observed in guinea pigs sensitized and then challenged with the zonal purified vaccine. Again, guinea pigs sensitized with either whole duck embryo extract or cell culture vaccine were negative for anaphylaxis when challenged with the zonal purified vaccine. Hence, this 10% experimental whole duck embryo vaccine was responsible for all observed anaphylaxis reactions, whether homologous or heterologous.

**DISCUSSION**

The above experiments indicate that a duck embryo cell culture vaccine containing $10^{4.4}$ to $10^{4.9}$ logs of virus can be concentrated from volumes of 14 to 30 liters to volumes of 400 to 600 ml with peak virus titers 100 to 1,000 times greater than those of the starting material. The

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**TABLE 2. Rabies purified cell culture vaccines**

| Lot no.  | Amt of vaccine | Original LD50 | Peak fraction LD50 |
|----------|----------------|---------------|-------------------|
| T-61639  | 14             | 4.3           | 6.50              |
| T-61728-A | 20         | 4.4           | 6.40              |
| T-61728-B | 20         | 4.4           | 6.40              |
| T-65148  | 30             | 3.0           | 6.30              |
| T-65149  | 30             | 3.0           | 6.30              |

* LD50: log10/0.03 ml of vaccine inoculated intracerebrally in adult white mice.

**TABLE 3. Potency testing of rabies cell culture vaccines (prior to purification)**

| Lot no.  | LD50 | Vaccine | Protein Nt (mg/ml) | NIH ratio | Habel index |
|----------|------|---------|-------------------|-----------|-------------|
| T-61639-A | 4.5  | Liquid  | 0.72              | 0.23      | 269,000     |
| T-61639-B | 4.5  | Lyophilized | 0.72         | 0.18      | 269,000     |
high-titer fractions containing $10^{4.5}$ to $>10^{6.9}$ logs/0.03 ml of vaccine can then be diluted back to about 10 times the original volume. The resulting products had very low protein nitrogen, and three of five lots passed the NIH potency test when tested as being equal in protein nitrogen to a 10% duck embryo vaccine or the NIH reference; these three lots induced good seroconversion in rabbits after one dose of vaccine.

However, the high-titer fractions could also be combined and inactivated without dilution, which would result in a cell culture vaccine of high titer (greater than $10^{6.8}$). This type of vaccine might be very useful for immediate treatment of human subjects exposed to rabies by severe wild animal bites. This is the type of vaccine we envision for future postexposure treatment.

The zonal purified vaccine induced good SN antibody in rabbits after a single 1.0-ml dose of vaccine containing only 0.01 mg of protein nitrogen/ml. Both the standard and zonal-purified liquid vaccines (Fig. 5) contained an AlPO₄₄ adjuvant; yet antibody response was prompt with both vaccines—in fact, within 5 days with the liquid zonal-purified product.

### TABLE 4. NIH potency test results with rabies purified cell culture vaccines

| Lot no.       | Type of vaccine | LD₅₀ (mg/ml) | Protein N (mg/ml) | Sucrose concn | NIH potency ratio |
|---------------|-----------------|--------------|-------------------|---------------|------------------|
| T-61728-A     | Liquid          | 4.0          | 0.01              | 6             | 0.279            |
| T-61728       | Lyophilized     | 4.0          | 0.01              | 6             | 0.373            |
| T-65148       | Lyophilized     | 3.9          | 0.018             | 5             | 0.550            |
| T-65149-D     | Lyophilized     | 4.3          | 0.0504            | 10            | 0.42             |
| T-65149       | Lyophilized     | 3.5          | 0.0104            | 6             | 0.052            |

* NIH potency ratio of 0.30 necessary to pass test.

![Graph showing SN titers over days post immunization](image)

**FIG. 6.** Immune response in rabbits to various duck embryo rabies vaccines. (A) Cell culture vaccine, liquid. (B) Cell culture vaccine, lyophilized. (C) Purified cell culture vaccine, liquid. (D) Purified cell culture vaccine, lyophilized.

### TABLE 5. Anaphylactic shock in guinea pigs: controls and homologous reactions

| Sensitized with                        | Challenged with                        | Animals reacting/total no. of animals |
|----------------------------------------|----------------------------------------|---------------------------------------|
| ---                                    | 10% Experimental duck embryo vaccine   | 0/2                                   |
| ---                                    | Cell culture vaccine                    | 0/2                                   |
| ---                                    | Zonal-purified cell culture vaccine     | 0/2                                   |

### TABLE 6. Anaphylactic shock in guinea pigs: heterologous reactions

| Sensitized with                        | Challenged with                        | Animals reacting/total no. of animals |
|----------------------------------------|----------------------------------------|---------------------------------------|
| 10% Experimental duck embryo vaccine   | Cell culture vaccine                    | 0/2                                   |
| 10% Experimental duck embryo vaccine   | Zonal-purified cell culture vaccine     | 0/2                                   |
| Cell culture vaccine                   | Zonal-purified cell culture vaccine     | 2/2*                                  |
| Zonal-purified cell culture vaccine    | Zonal-purified cell culture vaccine     | 0/2                                   |
| Zonal-purified cell culture vaccine    | Cell culture vaccine                    | 0/3                                   |

* Slight transitory congestion.

However, the standard cell culture and zonal-purified lyophilized vaccines (Fig. 6) exhibited rather a long latent period before the induction of antibody. This delayed response on the part of the lyophilized products may be at least partially explained by the fact that the diluent used to reconstitute these products contained the adjuvant. The vaccines were used immediately after reconstitution, which perhaps did not permit adequate time for viral antigen adsorption to the adjuvant. Also, antibody responses are frequently delayed with adjuvant-treated vaccines.

Nonetheless, the fact remains that the zonal-purified vaccines held up well in the NIH test developed for vaccines with heavy tissue debris. Also, both liquid and lyophilized zonal-purified vaccines induced good seroconversion in rabbits after only one dose of vaccine.

A 10% suspension of experimental duck embryo vaccine induced severe anaphylactic...
shock in sensitized guinea pigs, whereas guinea pigs sensitized with the cell culture vaccine or zonal purified vaccines were negative for anaphylactic shock when challenged with the homologous vaccine. Also, all heterologous reactions were negative for anaphylaxis, except for a slight transitory congestion noted when the 10% experimental duck embryo vaccine was used as the challenge. The experimental 10% whole duck embryo vaccine containing the entire spectrum of duck antigens was responsible for all anaphylaxis reactions whether homologous or heterologous. The cell culture and zonal-purified duck embryo vaccines, both relatively free from foreign duck antigens, were notable for their freedom from anaphylactogenic activity both in homologous and heterologous combinations.

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