Recovery of Protective Activity in Rabies Virus Vaccines Concentrated and Purified by Four Different Methods

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Rabies vaccines concentrated by ultrafiltration, zinc acetate precipitation, ammonium sulfate precipitation, or aluminum phosphate gel adsorption were compared with respect to recovery of protective activity and purity, as measured by protective activity per mg of protein. Vaccine obtained by ammonium sulfate precipitation had a better recovery rate and a higher purity than those prepared by the other methods. Potent vaccines were also obtained by the zinc acetate precipitation and aluminum phosphate gel adsorption methods, whereas ultrafiltration was the least satisfactory method from the standpoint of vaccine purity. Chromatography of virus concentrated by ultrafiltration on a cellulose ion exchange column reduced the level of nonviral proteins. The protective activity data obtained for the vaccines examined in these experiments were found to correlate with the vaccine's complement fixation titer per mg of protein.

In previous reports from our laboratory we have shown that rabies virus may be successfully concentrated by an ultrafiltration method (21) or by precipitation with zinc acetate (15). After inactivation with β-propiolactone (BPL), these virus concentrates have proven to be potent vaccines (21). We have also found that rabies virus and other rhabdoviruses may be efficiently concentrated by ammonium sulfate precipitation (1). Recently Schneider et al. (11) used the aluminum phosphate gel adsorption technique (9) to concentrate and purify rabies virus. Rabies virus thus concentrated was also an efficient immunogen.

The experiments described in this paper were performed to compare the usefulness of each of these four different concentration and purification techniques in producing a rabies vaccine from virus which had been propagated in tissue culture and then inactivated with BPL. In judging each of these methods we have emphasized three points: (i) ease of manipulation; (ii) recovery of protective activity as compared to initial virus-containing tissue culture fluids; and (iii) effectiveness of purification as measured by the antigenic value of the vaccine per mg of protein. In addition, we have evaluated the protective activity of rabies virus inactivated with BPL before concentration by ultrafiltration in comparison with that inactivated after concentration.

MATERIALS AND METHODS

Virus and cells. The tissue culture-adapted Pitman Moore strain of rabies virus (20) was used for all vaccine preparations; the CVS strain, propagated in mouse brain, was used as challenge virus in vaccine potency tests. Baby hamster kidney (BHK-21/13S) cells (7) and cells of human diploid strain WI-38 (4) were propagated according to methods previously described (21). BHK-21/13S cells propagated in roller bottles were infected with rabies virus as described by Sokol et al. (15) and then refed with Eagle minimum essential medium (MEM) containing 0.1% (v/v) bovine serum albumin and 0.44% (v/v) sodium bicarbonate. The serum albumin was added to the medium to stabilize the biological activity of progeny virus. Virus was recovered from supernatant tissue culture fluid after 96 hr of incubation at 33 C. When WI-38 cells were employed for virus propagation, the method of Wiktor et al. (21) was followed, and the infected cells were refed with medium containing either 10% (v/v) inactivated calf serum or 0.1% (v/v) human serum albumin. No diethylaminoethyl (DEAE)-dextran was used to facilitate the infection of either cell system.

Inactivation of virus infectivity. BPL was added to virus-containing fluids to make a final concentra-
of 0.025% (v/v), and the fluids were maintained at 4 °C for 20 hr, and then at 37 °C for 2 hr (21). The absence of residual infectious virus was determined by intracerebral inoculation into mice (see below).

Concentration methods. Rabies virus-infected tissue culture fluid was freed of cellular debris by low-speed centrifugation (750 × g for 30 min), inactivated by BPL, and concentrated by one of the methods described below. In all cases, the unconcentrated vaccine was kept at 5 °C during manipulation.

Method 1. Viral and subviral particles were concentrated by ultrafiltration in a specially designed filtration apparatus (17), employing nitrocellulose membranes with a porosity of 10 to 20 nm (Filter SM12 133, Membranafiltergesellschaft mbH, Göttingen, Germany) as previously described (21). After concentration the fluid was clarified by low-speed centrifugation; the product is referred to in this paper as the ultrafiltration concentrated virus (UCV).

Method 2. Virus was precipitated by zinc acetate by the method of Sokol et al. (15). Precipitation was achieved by the addition of zinc acetate to a concentration of 0.02 M, precipitated virus was recovered by centrifugation (1,000 × g for 90 min), and the pellet was resuspended in a saturated solution of disodium ethylenediaminetetraacetate (EDTA), pH 7.8. The volume of the EDTA solution used for dissolving the precipitate was 3 to 5% of that of the original culture fluid. After clarification, the potential vaccine was filtered through a column of Sephadex G-75 which had been equilibrated with 0.13 M NaCl, 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-chloride (NT buffer), pH 7.8 EDTA and zircon ions were effectively removed by this procedure.

Method 3. Virus was precipitated by the ammonium sulfate method of Aaslestad et al. (1). Calf serum (to 2%, v/v) and ammonium sulfate (30%, w/v) were then added as co-precipitants for the virus. The pH was maintained at 7.5 by the addition of 1 M Tris, pH 8.0, and the precipitated material was recovered by centrifugation as described above. The pellet was dissolved in a minimal volume NT buffer plus 0.01 M EDTA and freed of particulate matter by low-speed centrifugation.

Method 4. Batch adsorption on aluminum phosphate gel, by the method of Schneider et al. (11), was also used to concentrate and purify rabies virus for vaccine use. Freshly prepared gel (9), equilibrated with 0.05 M phosphate buffer, pH 7.1, was mixed in the recommended ratio (11) with clarified tissue culture fluid containing inactivated rabies virus. After being washed twice with 0.05 M phosphate buffer, the virus was eluted from the gel with 0.3 M phosphate buffer, pH 6.0.

After concentration by each of the above methods, rabies virus was sedimented by centrifugation in a no. 30 Spinco rotor at 35,000 × g for 90 min. The sediment was resuspended in 1.0% of the original culture fluid volume in phosphate-buffered saline with the aid of brief sonication in a Raytheon sonic oscillator (1 min at 10 kc). A final low-speed centrifugation was used to remove particulate matter. The inactivated virus concentrate thus obtained was stored at −70 °C until assayed for biological activities.

Assay procedures. Rabies virus infectivity was quantitated by plaque titration on agarose-suspended BHK-21/13S cells and expressed as plaque-forming units (PFU) per ml (13). Inactivated, undiluted vaccine preparations were assayed for virus inactivation by intracerebral inoculation (0.03 ml) into groups of 10 4-week-old mice. Titrations of rabies-specific complement-fixing (CF) antigen and hemagglutinin (HA) were carried out by microtechnique, according to published procedures (5, 15). Specific activity values of CF and HA were expressed in terms of the reciprocal of the titer per mg of protein.

Vaccines were evaluated for their protective activity by the National Institutes of Health (NIH) potency test (14). Antigenic values (AV) were expressed as the ratio of the dilution, of the vaccine under test protecting 50% of mice (ED50), to that of standard NIH reference vaccine lot no. 175. The degree of vaccine purity was expressed in terms of AV per mg of protein. All recovery values were calculated as percentages by multiplying the reciprocal of the CF or HA titer or the AV by the volume of tested material in comparison with the starting material, which was defined as unity.

Protein was determined by the method of Lowry et al. (6).

Chromatography on DEAE-cellulose. Column chromatography on DEAE-cellulose was performed according to the method of Fucaldo et al. (3) except that the pH of the elution buffer was 8.2.

RESULTS

A pool of rabies virus, propagated in BHK-21/13S cells and having an infectivity titer of 107.5 PFU/ml, was inactivated by BPL. The CF titer of the unconcentrated vaccine was 1:8 per 0.05 ml, whereas its AV was 1.96 per ml. The pool was divided into four portions, each of which was then concentrated by a different method. As represented in Table 1, the high-speed sediment obtained after ultrafiltration contained considerably more protein than the vaccines prepared by the precipitation or adsorption techniques. This was due to the membranes’ nondiscriminatory retention of essentially all particulate material and macromolecules of greater than 20,000 molecular weight. Serum albumin was retained under such conditions and was detected as a contaminant in the high-speed sediment. The recovery of CF activity in each concentrate was quite low as compared to the values obtained by Wiktor et al. (19) and Schlumberger et al. (10) for similar preparations. Nevertheless, a marked increase in the specific CF activity was observed in each of the vaccines, especially in those prepared by precipitation or adsorption methods. High-speed sediments prepared after ammonium sulfate precipitation or aluminum phosphate gel adsorption showed
CF activity values 20- to 21-fold higher than unconcentrated tissue culture fluid. When the purity and recovery of protective activity were determined (Table 1), the ammonium sulfate-concentrated vaccine was found to be superior to the other vaccines tested.

We next attempted to employ ammonium sulfate precipitation to recover rabies protective activity from a pool of low-titer virus (10^4.8 PFU/ml) which had been propagated in WI-38 cells in the presence of 10% calf serum. Table 2 summarizes the results of this experiment. Although no CF activity was detected in the unconcentrated virus pool, the vaccine concentrate (×580) had a specific CF activity of 145.5/mg of protein. When the protective activity was determined, it was found that the AV per mg of protein had increased 100-fold over the value of the unconcentrated vaccine and that 29% of the protective activity was recovered. The specificity of this concentration and purification procedure for rabies vaccine production was proven, because only 0.3% of the protein content initially present was found in the vaccine concentrate.

Even though rabies vaccine concentrated by ultrafiltration and high-speed centrifugation was judged least satisfactory as far as purity was concerned (Table 1), we felt that this method might be useful if applied together with a secondary separation step in which proteins from serum albumin or whole calf serum could be removed from the vaccine. To achieve this goal, we adapted the DEAE-cellulose chromatographic method of Fuscaldo et al. (3). We were encouraged because trial experiments showed that bovine serum albumin was quantitatively eluted when the column was developed with 0.15 m NaCl, whereas virus was completely retained on the column. The data

**Table 1. Recovery of protective activity from inactivated rabies virus vaccine concentrated by different procedures**

| Concen procedure* | Volume concn factor | Protein concn (mg/ml) | Complement fixation* | Protective activity* |
|-------------------|---------------------|-----------------------|----------------------|----------------------|
|                   |                     |                       | Specific activity (CF/mg protein) | Recovery (%) | Specific activity (AV/mg of protein) | Recovery (%) |
| Unconcentrated tissue culture fluid | ×1 | 1.96 | 82 (100) | 1.0 (100) |
| Ultrafiltration | ×100 | 5.65 | 377 | 6.6 | 12.4 | 35.0 |
| ZN(CH_3CO)_{2} precipitation | ×100 | 0.27 | 1,485 | 2.5 | 237.0 | 32.5 |
| (NH_4)SO_4 precipitation | ×100 | 0.24 | 1,670 | 3.3 | 415.0 | 51.0 |
| (Al){_2}PO_4 adsorption | ×100 | 0.23 | 1,740 | 3.3 | 387.0 | 33.5 |

*An 8.6-ml amount of rabies virus propagated in BHK cells and having an infectivity titer of 10^4.8 PFU/ml before inactivation was divided into four portions and concentrated as shown. Concentrated samples had been centrifuged as described in Materials and Methods.

**Table 2. Concentration of rabies virus by the ammonium sulfate precipitation method**

| Sample | Volume (ml) | Protein concn (mg/ml) | Complement fixation | Protective activity* |
|--------|-------------|-----------------------|---------------------|----------------------|
|        |             |                       | Specific activity (CF/mg protein) | Specific activity (AV/mg of protein) | Recovery (%) |
| Unconcentrated tissue culture fluid* | 14,000.0 | 9.6 | <4.2 | 0.020 | (100) |
| Virus concentrate | 23.5 | 16.5 | 145.5 | 2.0 | 29 |

*The unconcentrated virus pool protected mice at an ED_{50} dilution of 2.5; the NIH reference rabies vaccine no. 175 had an ED_{50} dilution of 13.2, and thus an antigen value (AV) of 0.19 was calculated. A 63 LD_{50} of rabies strain CVS was used as challenge.

* Rabies virus pool contained 10^4.8 PFU/ml before β-propiolactone inactivation. Virus was propagated in WI-38 cells in the presence of 10% calf serum.
shown in Fig. 1 are representative of numerous separations of bovine serum albumin from rabies virus that have been performed. These data were obtained when a live preparation of UCV (×40) of BHK-21/13S-propagated virus with a titer of 10^8.2 PFU/ml was chromatographed. Prior to chromatography the virus preparation was dialyzed against five volumes of distilled water in order to lower the total salt content to less than 0.05 M. Rabies-specific antigen was detected by HA titration and, as shown in Fig. 1, only that material eluted by 0.7 M NaCl in Tris-succinate (0.05 M, pH 8.2) contained HA activity. Three fractions (51 to 53) contained 65.7% of the HA activity applied to the column, but only 24.5% of the protein. Two fractions were opalescent and could easily be distinguished from neighboring fractions by their ability to scatter light. The small shoulder of 280-nm absorbing material, on the trailing side of the material eluting with 0.7 M NaCl, was found to be residual phenol red carried over from the tissue culture medium.

Table 3 presents data which reflect the degree to which the UCV vaccine had been freed of contaminating protein by DEAE chromatography and the extent to which viral HA and protective activity were retained. The protein concentration in the vaccine preparation was reduced 83.2%, whereas the specific HA titer increased approximately 3.3-fold. On the other hand, a less adequate rabies protective antigen was obtained, because the AV per mg of protein value decreased from 2.43 to 1.43 after chromatography. Whether DEAE chromatography will prove to be a useful step in processing rabies UCV vaccine must depend on the degree of recovery of the protective activity of the vaccine thus obtained. There is little doubt that this method can remove most of the serum albumin from an UCV. The low specific AV in the purified vaccine may be explained by the fact that BPL inactivation took place after the bulk of the protein was removed. Therefore, the effect of the concentration of protein in a vaccine sample on the loss of protective activity during BPL inactivation was next determined.

The following experiment was performed to determine the effect of protein content on the retention of protective activity during BPL inactivation. A volume of 1,850 ml of rabies virus-containing tissue culture fluid (infectivity titer, 10^8.5 PFU/ml; 0.1% human serum albumin) propagated in WI-38 cells was divided into two portions. One portion (low protein content) was inactivated with BPL and then concentrated by ultrafiltration. The second portion was first concentrated by ultrafiltration (high protein content) and then inactivated by BPL. Protein content, CF, and protective activity were determined for both vaccines and compared with that of unconcentrated vaccine (inactivated at low protein content). The data are summarized in Table 4. The protein content of the UCV increased proportionally with the concentration factor. Approximately 7 to 9% of the total protein was present in the fluids which passed through the membranes. The specific CF activity remained almost constant, reflecting the nearly complete retention of CF antigen. On the other hand, the AV per mg of protein of the UCV treated with BPL before concentration (low protein content) was only one-half that of the unconcentrated fluid. In addition, only about 40% of the protective activity was recovered. When BPL was used to inactivate a live UCV vaccine, in which case protein was about 13 times as concentrated, a recovery of 77.5% of the protective activity was obtained, and the vaccine's specific protective activity was 20% greater than that of the unconcentrated tissue culture fluid.

Finally, Fig. 2 shows that a close correlation exists between the specific CF activity and the specific protective activity of a given vaccine. This confirms the previous report of Wiktor et al. (21) which showed that a plot of the log_{10} of the reciprocal of the CF titer versus the log_{10} of the ED_{50} value of rabies vaccine resulted in a straight line. Data plotted in Fig. 2 have been taken from the three experiments sum-

![Fig. 1. DEAE chromatography of an ultrafiltration concentrated virus preparation of rabies virus. An 8-ml rabies concentrate, containing 33.0 mg of protein per ml and 8.2 × 10^8 hemagglutinin units, was applied to a 2.3- by 20.0-cm DEAE column. The column was developed with the indicated buffers at a flow rate of approximately 2 ml/min. Each fraction contained 4.5 ml.](http://aem.asm.org/)
TABLE 3. Protective activity of DEAE-chromatographed ultrafiltration concentrated (UCV) rabies virus

| Sample          | Volume (ml) | Protein concn (mg/ml) | Hemagglutination | Protective activity* |
|-----------------|-------------|----------------------|------------------|---------------------|
|                 |             |                      | Specific activity (HA/mg of protein) | Recovery (%) | Specific activity (AV/mg of protein) | Recovery (%) |
| UCV             | 8.0         | 33.0                 | 29.5             | (100)              | 2.43              | (100) |
| DEAE purified virus* | 9.0         | 4.7                  | 98.0             | 62                 | 1.43              | 13.6 |

* Rabies virus ultrafiltrate (×40) protected mice at an ED₅₀ dilution of 2,000; the NIH reference rabies vaccine no. 175 had an ED₅₀ dilution of 25, and thus an antigen value (AV) of 80 was calculated. A 30 LD₅₀ of rabies strain CVS was used as challenge.

TABLE 4. Effect of β-propiolactone (BPL) inactivation on the protective activity of rabies virus ultrafiltration concentrated vaccine (UCV)

| Sample                      | Volume concn factor | Protein concn (mg/ml) | Complement fixation* | Protective activity* |
|-----------------------------|---------------------|-----------------------|----------------------|---------------------|
|                             |                     |                      | Specific activity (CF/mg of protein) | Recovery (%) | Specific activity (AV/mg of protein) | Recovery (%) |
| Unconcentrated tissue culture fluid* | ×1                   | 2.4                  | 34                   | (100)              | 0.10              | (100) |
| UCV treated with BPL before concn | ×20.9               | 38.5                 | 33                   | 72.0               | 0.05              | 39.8 |
| UCV treated with BPL after concn | ×20.7               | 32.0                 | 40                   | 77.0               | 0.12              | 77.5 |

* Unconcentrated rabies virus-containing tissue culture fluid had a CF titer of 1:4 per 0.05 ml. This material protected mice at an ED₅₀ dilution of 8.3; the NIH reference rabies vaccine no. 175 had an ED₅₀ dilution of 36.0, and thus an antigen value (AV) of 0.23 was calculated. A 21 LD₅₀ of rabies strain CVS was used as challenge.

* A 1,850-ml amount of rabies virus, propagated in WI-38 cells, having an infectivity titer of 10⁶ PFU/ml was divided into two portions and inactivated with BPL either before or after concentration by ultrafiltration. These points were obtained for vaccine preparations prepared from virus propagated in WI-38 and BHK-21/13S cells in medium containing either bovine or human serum albumin or calf serum. Moreover, they are representative of both unconcentrated vaccine and vaccine concentrated by various methods.

DISCUSSION

Each of the bulk concentration methods was rapid and reasonably efficient in terms of the percentage of recovery of protective activity. Each method, however, had certain strengths and weaknesses. Ultrafiltration required little handling of the virus-containing fluid and no centrifugation after the initial clarification until the UCV was sedimented in the ultracentrifu-
forces. This method, however, was the least selective in that it concentrated all material greater than approximately 20,000 molecular weight. The precipitation and adsorption methods each required the introduction of chemical reagents, in addition to tissue culture constituents, and also from one to three centrifugations at moderately high centrifugal forces. On the other hand, they yielded vaccine preparations considerably enriched in rabies protective activity.

The ammonium sulfate precipitation procedure produced a vaccine with the highest protective activity, as compared to the other concentration methods evaluated in this report (Table 1). Use of ammonium sulfate precipitation followed by high-speed centrifugation resulted in a vaccine in which 51% of the protective activity present in unconcentrated vaccine could be recovered and which was purified 415-fold. The specific protective activity of this vaccine compared well with previously reported rabies vaccines prepared in our laboratory.

Wiktors et al. (21) reported that zinc acetate vaccine (prepared in a manner identical to those used in this study) could protect mice to the extent of 550 AV/mg of protein; however, their vaccine was BPL inactivated in the concentrated state. The efficient concentration and purification of an active vaccine from a low-titer rabies virus pool containing 10% calf serum are added evidence of the value of ammonium sulfate precipitation as a concentration and purification method (Table 2).

Concentration methods employing zinc acetate precipitation or aluminum phosphate gel adsorption also yielded vaccines of adequate recovery of protective activity and of purity. Ultrafiltration was far less effective for the preparation of a vaccine of increased purity because of the indiscriminate nature by which the membrane held back all particles and most macromolecules. Ultrafiltration, however, did permit an adequate recovery of protective activity. The specific CF activities, shown in Table 1, for vaccine concentrates prepared by precipitation or adsorption are 18- to 20-fold greater than that of unconcentrated vaccine, but are still far below the values reported for purified rabies virus (11, 15). The low recovery of CF activity was probably due to the fact that soluble antigens which fix complement (10) had been removed during high-speed centrifugation. Proof of this was found when a recovery of 75% of the CF activity was determined in the UCV before high-speed centrifugation (data not shown).

The results obtained with rabies vaccine concentrated by aluminum phosphate gel adsorption are in slight disagreement with those reported by Schneider et al. (11, 12). Our rabies concentrates had a lower specific CF activity; however, differences in the procedure used to quantitate protein may have caused this discrepancy (11). Differences between the protective activity of aluminum phosphate gel-concentrated rabies vaccine prepared by Schneider et al. (12) and that reported in this paper may be due to differences in the strain of rabies virus used and to their virus concentrating being further purified by density gradient centrifugation.

The separation of serum albumin from rabies virus was readily performed by DEAE chromatography (Figure 1). The chromatographic method employed was convenient to use and could be "scaled up" to receive samples containing large amounts of protein. As may be seen in Table 3, a significant increase in specific HA activity, as well as a good overall recovery of HA activity, was achieved after DEAE chromatography. Although ion exchange celluloses have been utilized in the purification of rabies virus with conflicting results (18, 19), the DEAE chromatography method reported here has been successfully used by McSharry and Wagner (8) for the purification of vesicular stomatitis virus. The apparent loss of protective activity was considered to be due to excessive treatment with BPL in an environment low in protein content. Although it is accepted that the lethal effect of BPL is a result of alkylation of imidazole functional groups in the viral ribonucleic acid (2), the possibility exists that considerable alkylation of sensitive functional groups of rabies virus surface protein or glycoprotein (16) could destroy protective activity. In the absence of large amounts of nonviral protein, partially purified rabies vaccine might be especially susceptible to such alteration. Experiments are under way to determine the effect of added protein on the stability of rabies vaccine protective activity.

The data (Table 4) also suggest an increased loss of protective activity when BPL is used to inactivate infectious virus in a medium of low protein content. If safety considerations for laboratory personnel require vaccine inactivation prior to concentration, the observed loss may be unavoidable. Complete interpretation of these data is difficult because the control unconcentrated tissue culture fluid and the sample inactivated before concentration differed only in that the latter was concentrated.
20-fold before assay for AV. Whether the control sample in this experiment was itself rendered a less potent protective vaccine cannot be determined from these data. An explanation for the reduced protective activity in the vaccine preparation concentrated after inactivation may be that particle fragmentation occurred and protective activity was lost by adsorption onto elements of the filtration unit.

The apparent correlation between rabies vaccine-specific CF activity and specific protective activity (Fig. 2) is judged significant in that data from other vaccine preparations prepared in our laboratory fall within the limits of the described line. A complete understanding of this relationship must await additional experimentation. This correlation should be accepted only with caution because the presence of rabies soluble antigen having CF activity has been proven (10) and, therefore, high CF titers would bias the correlation in unpurified vaccine preparations. In addition, recent experiments in our laboratory have shown that rabies-infected WI-38 cells maintained in medium containing human serum albumin for long periods of time can produce high levels of CF antigen, whereas little infectious virus or protective activity can be detected (H. F. Clark and T. J. Wiktor, unpublished data).

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