Inactivated Rabies Vaccine Produced from the Flury LEP Strain of Virus Grown in BHK-21 Suspension Cells

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Suspension cultures of BHK-21 cells maintained at 32 to 33°C were infected with the Flury LEP strain of rabies virus. By using a cell concentration of $2.0 \times 10^6$ to $2.5 \times 10^6$ cells per ml infected at a multiplicity of 0.05, high titers of extracellular virus were reached in 96 to 120 h, and potent inactivated vaccines were prepared from culture fluids harvested between 96 to 168 h. The addition of 1% bovine serum to the maintenance medium resulted in an increase in virus yields and vaccine potency. Estimation of the number of infected cells by immunofluorescent procedures proved a rapid and reliable guide to the virus content of suspension cultures.

The growth of rabies virus in tissue culture has simplified the large-scale production of antigen for both attenuated and inactivated vaccines for veterinary use. Hamster kidney cells have been used by a number of workers. Thus, Petermann et al. (8), using the NIL line from the Wistar Institute, prepared an inactivated vaccine from a fixed rabies strain. More recently, Crick and Brown (5) showed that the Flury LEP strain of virus grown in BHK-21 clone 13 (7) cell monolayers and inactivated with acetylated ethyleneimine (AEI) (2) was also an excellent immunogen for dogs and cattle. Atanasiu et al. (1) found that the Pasteur strain of rabies virus could be grown in suspended BHK-21 cells and used as an inactivated or attenuated vaccine.

In this paper we examine some of the requirements for the production of potent inactivated rabies vaccines from the LEP strain of virus grown in BHK cell suspension cultures.

MATERIALS AND METHODS

**BHK suspension cells and virus strain.** The BHK suspension cell strain and the methods of routine culture have been described previously (3, 4). The Flury LEP strain of rabies virus grown in BHK-21 cell monolayers (5) was passaged five times in suspension culture, mixed with an equal volume of sterile glycerol, and stored at $-20$°C. This stock virus, which had an infectivity titer of $10^4.5$ (mean lethal dose) LD$_{50}$/ml, was used for all experiments.

**Culture vessels and virus production.** Initially, virus was grown in 250-ml cultures in the cylindrical Pyrex glass vessels and laboratory apparatus described by Smith and Burrows (9). The pH was maintained by a continuous flow of 5% CO$_2$-air mixture across the surface of the culture at a flow rate of 25 ml/min.

Experimental results obtained in these vessels were later confirmed in 1-liter culture vessels fitted with automatic pH and temperature recording and controlling equipment similar to that described by Telling and Elsworth (10). The pH was controlled at the desired value $\pm 0.05$ pH unit, and the temperature was controlled at the desired value $\pm 0.5$°C.

The 1-liter vessels were sterilized in situ by direct steam injection at 5 lb/in$^2$ for 4 h. Effluent air from virus-infected culture vessels was passed through a laboratory tube furnace operating at a temperature of 600° C. This was a safety device to insure the inactivation of any virus carried over from the culture vessels.

The maintenance medium consisted of eight parts of Eagle basal medium modified to contain 3.3 g of sodium bicarbonate per liter and twice the normal concentration of amino acids and vitamins, one part of 2% (wt/vol) tryptose phosphate broth, and Seitz (EK) filtered adult bovine serum to a final concentration of 1% (wt/vol) except where indicated.

BHK suspension cells were collected by low-speed centrifugation and resuspended in maintenance medium at the desired concentration.

Glycerinated stock virus was added to the culture to give a virus multiplicity of 0.05; the volume of virus inoculum added was approximately 1% of the total culture volume.

Virus-infected cultures were maintained at 32 to 33°C and were stirred at a speed of 300 rpm with the pH controlled at 7.4. Cell counts were made every 24 h, and the number of viable cells was enumerated by trypan blue permeability.

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Assay of infectivity. Virus was assayed by the intracerebral inoculation of 7-day-old mice by using a 10-fold dilution series and five mice per dilution. The volume inoculated was 0.03 ml, and the diluent was phosphate-buffered saline (PBS) containing 1% ox serum.

The number of paralyzed and dead animals was recorded daily over a period of 10 days, and the 50% end points were calculated by the method of Kärber (6).

Enumeration of infected cells. Samples of suspension culture were centrifuged; the cellular deposit was washed in PBS and, without prior fixation, resuspended in 0.2 ml of fluorescein-conjugated anti-rabies serum (obtained from the Pasteur Institute, Paris) and incubated for 1 h at 37 C. After further washes in PBS, the cells were resuspended in buffered glycerol and mounted with cover slips on microscope slides. The number of fluorescing cells was counted by using a Reichart Binolux microscope (exciter filter BG 13/6 mm and barrier filter C-G9/11 mm), and the percentage of infected cells was estimated from counts of 300 to 500 cells.

Inactivation of virus. After removal of cells and cell debris by centrifugation, virus was inactivated by the addition of AEI to a final concentration of 0.05% at a temperature of 37 C for 6.5 h. The excess AEI was destroyed by adding 0.1 vol of 20% sodium thiosulphate. Innocuity tests of the inactivated virus were carried out by the intracerebral inoculation of at least 50 7-day-old mice (5).

Estimation of immunogenic activity. The level of antibody produced by a single inoculum of vaccine in 6- to 8-week-old mice was used as a measure of vaccine potency (5). Appropriate dilutions of the inactivated vaccine were inoculated, in 1-ml amounts, intraperitoneally into groups of mice (five mice per dilution). After 28 days the animals were bled, and the serum was separated and stored at −20 C until required. Individual sera were tested for specific neutralizing antibody by mixing 10-fold dilutions of virus with an equal volume of a 1:10 dilution of serum (heat inactivated at 56 C for 30 min) and incubating at room temperature for 60 min; 0.03-ml volumes of the mixture were then inoculated intracerebrally into 7-day-old mice. Control mixtures of virus dilutions with 1:10 dilutions of normal serum and standard hyperimmune serum were included in each test. The depression in virus titer produced was taken as the neutralizing activity or index (NI) of 0.015 ml of a 1:10 dilution of serum.

RESULTS

Effect of temperature on cell viability and virus yield. In suspension cultures maintained at 36 C, a marked drop in cell viability occurred after 48 to 72 h (Fig. 1), and under these conditions the peak infectivity of virus-infected cultures seldom exceeded $10^4$ to $10^5$ LD$_{50}$/ml. When cell cultures were maintained at 33 C, the cells remained viable for longer periods (Fig. 1) and consistently yielded virus concentrations greater than $10^7$ LD$_{50}$/ml.

Effect of cell concentration on virus yield and the potency of vaccines. Cultures were infected at a multiplicity of 0.05. The effect of cell concentration on the extracellular virus titers is given in Fig. 2 for the range of $0.5 \times 10^4$ to $2.5 \times 10^4$ cells/ml. The highest infectivity titers were obtained with cell concentrations of $2.5 \times 10^4$ cells/ml. The antibody responses of mice to vaccines prepared from virus grown at different cell concentrations are listed in Table 1. The immunogenicity of the prepared vaccines increased as the cell concentration increased.

Optimal time of harvest. The antibody responses of mice to vaccines prepared from virus harvested at 96, 120, 144, and 168 h are recorded in Table 2. Peak infective virus titer was reached at 120 h after infection of the cells, with the maximal immunogenicity of prepared vaccines occurring 24 to 48 h later.

Effect of serum. The inclusion of 1% ox serum in the maintenance medium resulted in a

![Fig. 1. Viability of BHK suspension cells maintained in culture at 33 and 36 C. Symbols: O, 33 C; *, 36 C.](image1)

![Fig. 2. Effect of cell concentration on the production of rabies virus. Symbols: O, $0.5 \times 10^4$ cells/ml; *, $1.5 \times 10^4$ cells/ml; ▲, $2.5 \times 10^4$ cells/ml.](image2)
two- to three-fold increase in virus yield (Table 3). A corresponding increase in the immunogenicity of vaccines prepared from the 168-h harvest of such cultures was also observed (Table 4).

To determine the effect of serum on the growth of virus, ox serum was added to a concentration of 1% (vol/vol) to virus-infected cultures at different times. Infective virus titers and the number of infected cells, as measured by immunofluorescence, are listed in Table 5. The peak infective virus titer was approximately 2- to 10-fold higher in cultures containing 1% ox serum, even when the serum was added as late as 96 h in the virus growth cycle. The maximal number of infected cells present in the culture containing 1% ox serum was about two- to three-fold higher than in cultures without serum.

Habel test. The potency of the suspension cell vaccine was determined for us by G. S. Turner, Lister Institute of Preventive Medicine, London, by using the Habel test. The titers of the challenge virus in mice vaccinated with a 1:10 dilution of vaccine and in unvaccinated mice were <10 and 10^4.8, respectively, equal to a Habel index of protection greater than 10^4.8 for undiluted vaccine.

Correlation of virus growth with the number of fluorescing cells. A good correlation was found between the growth of rabies virus in suspension cultures and the number of infected cells as measured by immunofluorescence (Fig. 3). The maximal number of cells that showed

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**Table 1. Antibody response of mice to vaccines prepared from virus grown at different cell concentrations**

| Cell concn (×10^6/ml) | Dilution of vaccine* | 1:1 | 1:5 | 1:25 | 1:125 |
|------------------------|----------------------|-----|-----|------|-------|
| 0.5                    | 3.2                  | 2.6 | 1.2 | 0.2  |       |
|                        | (3.1-3.7)            | (2.1-3.3) | (0.9-3.3) | (0.0-0.5) |       |
| 1.5                    | 3.7                  | 3.3 | 1.1 | 0.4  |       |
|                        | (3.2-4.2)            | (3.0-3.8) | (0.2-3.8) | (0.2-1.2) |       |
| 2.5                    | 4.2                  | 3.8 | 2.0 | 1.5  |       |
|                        | (3.5-4.4)            | (3.3-4.0) | (1.1-3.5) | (1.4-2.3) |       |

* Each group consisted of five mice; the geometric mean and the range of neutralizing indexes of 1:10 serum dilutions are given.

**Table 2. Comparison of the potency of rabies vaccines prepared at different time intervals of the virus growth curve**

| Time after infection (h) | Infective virus titer before inactivation (log_{10} LD_{50}/ml) | Dilution of vaccine* | 1:1 | 1:5 | 1:25 | 1:125 |
|--------------------------|---------------------------------------------------------------|----------------------|-----|-----|------|-------|
| 96                       | 6.8                                                           | 3.9                  | 3.2 | 1.3 | 0.6  |       |
|                          | (3.4-4.6)                                                     | (2.8-4.6)            | (0.8-2.2) | (0.2-1.8) |       |
| 120                      | 7.3                                                           | 3.8                  | 3.3 | 2.2 | 0.8  |       |
|                          | (3.3-4.3)                                                     | (2.5-3.9)            | (0.7-3.9) | (0.1-1.5) |       |
| 144                      | 7.0                                                           | 4.1                  | 3.5 | 2.0 | 1.5  |       |
|                          | (3.4-4.6)                                                     | (2.2-4.2)            | (1.8-3.4) | (0.8-2.8) |       |
| 168                      | 6.8                                                           | 4.4                  | 4.3 | 2.5 | 1.2  |       |
|                          | (4.2-4.6)                                                     | (4.2-4.4)            | (1.0-3.8) | (0.8-2.2) |       |

* Experiments were as in Table 1.

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**Table 3. Effect on virus titer of the presence of 1% ox serum in cultures infected with rabies virus**

| Time after infection (h) | Virus titer (log_{10} LD_{50}/ml) |
|--------------------------|-----------------------------------|
|                          | 1% ox serum | No serum |
|--------------------------|------------|----------|
|                          | 24         | 3.8      | 3.7      |
|                          | 48         | 5.4      | 5.6      |
|                          | 72         | 7.4      | 6.9      |
|                          | 96         | 7.5      | 7.1      |
|                          | 120        | 7.6      | 7.2      |
|                          | 144        | 7.5      | 7.0      |
|                          | 168        | 7.3      | 6.8      |

**Table 4. Effect on the immunogenicity of prepared vaccines of the presence of 1% ox serum in cultures infected with rabies virus**

| Serum content of vaccine | Dilution of vaccine* |
|-------------------------|----------------------|
|                         | 1:1  | 1:5  | 1:25 | 1:125 |
| 1%                     | 4.1  | (3.5-4.4) | (3.3-3.9) | (1.2-3.5) | (1.2-1.7) |
| Nil                    | 3.5  | (3.4-3.8) | (2.0-2.4) | (1.0-1.6) | (0.4-0.8) |

* Experiments were as in Table 1.

**Table 5. Effect of 1% ox serum on extracellular infective virus titer and the number of infected cells**

| Preparation  | Time after infection (h) | Infective virus titer (log_{10} LD_{50}/ml) | Infected cells (×10^6/ml) |
|--------------|--------------------------|---------------------------------------------|--------------------------|
| Serum free   | 96                       | 7.2                                         | 0.15                     |
|              | 120                      | 6.7                                         | 0.27                     |
|              | 144                      | 6.5                                         | 0.30                     |
| Serum added at 0 h | 96                       | 7.5                                         | 0.25                     |
|              | 120                      | 7.7                                         | 0.78                     |
|              | 144                      | 7.5                                         | 0.93                     |
| Serum added at 48 h | 96                       | 7.2                                         | 0.16                     |
|              | 120                      | 7.3                                         | 0.59                     |
|              | 144                      | 7.5                                         | 0.75                     |
| Serum added at 96 h | 96                       | 7.2                                         | 0.13                     |
|              | 120                      | 7.4                                         | 0.32                     |
|              | 144                      | 7.6                                         | 0.72                     |
fluorescence did not usually exceed 50% of the total cells in the culture.

**DISCUSSION**

The adaptation of BHK-21 cells to growth in suspension culture and the design and operation of an industrial-type culture system for the submerged growth of these cells have been described (4, 10). This type of culture system is now used extensively in the preparation of commercial inactivated foot-and-mouth disease virus (FMDV) vaccines.

The feasibility of producing an inactivated rabies vaccine by using the Flury LEP strain of virus grown in BHK-21 suspension cells has been studied. The culture vessels used were of 300-ml and 1-liter capacities; however, it is likely that the vaccine could also be produced in an industrial-type culture vessel of 30-liter capacity or more.

The results obtained here show that a controlled temperature of 32 to 33°C was necessary if good yields of virus were to be obtained. This appears to be related to the viability of the BHK suspension cells during the culture period.

Where large-scale production is envisaged, the volume of seed virus required to infect a culture is of considerable importance. BHK-21 suspension cells infected at a multiplicity of 0.05 or 0.5 showed no difference in the peak infective virus titer or in the immunogenicity of prepared vaccines. Multiplicities less than 0.05, however, gave lower infective virus titers, and the vaccines produced were of lower potency. By using a virus multiplicity of 0.05, 1 liter of seed virus containing 10^5 LD₅₀/ml can be used to seed a 100-liter culture vessel. This makes the production of seed virus from BHK-21 suspension cells or monolayer cells both feasible and economical.

The presence of 1% ox serum in the medium during the infective process was found to give a valuable increase in antigen titer. The role played by serum in the infective process is at present not fully understood, but it appears to be related to the number of cells that become infected.

Over the range of cell concentrations 0.5 × 10⁶ to 2.5 × 10⁶ cells/ml, infective antigen titers and immunogenicity of prepared vaccines increased as the cell concentration increased. The effect of still higher cell concentration on virus production was not determined, but Capstick et al. (3) found that with FMDV, complement-fixing antigen concentration increased as cell concentration increased, but not in direct proportion, and that a working compromise between efficiency and antigen titer obtained per milliliter was a cell concentration of 2.5 × 10⁶/ml.

The immunofluorescent method is routinely used as a diagnostic test for rabies. Its value lies in the speed at which a result may be obtained. The method, suitably adapted to the counting of infected BHK-21 suspension cells, has been shown to be of value in the early assessment of virus growth and as a guide to vaccine potency.

The levels of antibody produced in mice by a single inoculum of inactivated vaccine and the Habel index of protection of the vaccine prepared from virus grown in suspension culture were of the same order as those reported for inactivated vaccines produced from virus grown in BHK monolayer cells (5).

These results indicate that inactivated rabies vaccines for veterinary use can be prepared on a large scale by using the LEP strain of virus grown in suspension cultures of BHK-21 cells.

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