Persistence of Bacteriophages in Experimentally Infected Cell Cultures

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A bacteriophage, isolated from a live measles vaccine and designated φV-1₁₅, was intentionally introduced into cell culture systems. The bacterial virus persisted in the cell cultures after daily medium changes and trypsinization, and in cell lysates prepared by freezing and thawing. The results suggest that phages contaminating cell cultures may be carried over into the final product during the manufacture of viral vaccines.

The isolation of bacteriophages from fetal bovine sera intended for tissue culture use was described first by Merrill et al. (3) and subsequently was confirmed and extended by Chu et al. (2). The latter authors found no relationship between the presence of bacterial viruses and cell growth-promoting property of sera. More recently, Petricciani et al. (5) have isolated bacteriophages from certain lots of live virus vaccines prepared for human use. Because cell culture systems are widely used for vaccine production, they could have been one source of phages found in the final vaccine product.

Experiments described in this communication examined the effects of certain manipulations, which might be employed in vaccine production, on the persistence of phages after introduction into cell cultures.

MATERIALS AND METHODS

Bacteriophage. A phage designated as φV-1₁₅ was originally isolated from a lot of measles vaccine by using Escherichia coli C-3000 as the host bacterium (5). Phage T₅ was obtained from Miles Laboratories, Inc., Kankakee, Ill. Dilutions of stock phage suspension were made in tissue culture medium, and titers were determined just prior to inoculation of cell cultures.

Medium. A lot of fetal bovine serum aseptically drawn and found to be free from coliphages served as the 10% serum component in Eagle minimal essential medium without antibiotics.

Cell cultures. Cultures of primary chicken embryo and vervet monkey kidney cells were prepared by a method previously described (4). Diploid cell lines WI-38 (fetal human lung) and DBS-FRhL-2 (fetal rhesus lung) were used at passages 35 and 28, respectively. Primary cells were plated at a concentration of 3 × 10⁵/ml and the established cell lines at 10⁴/ml.

Inoculation and assay. Phages were added to complete Eagle minimal essential medium, which was then used as growth medium for the cell cultures. At the time of original plating, cell suspensions were mixed with 2.0 × 10⁴ or 1.1 × 10⁵ plaque-forming units (PFU)/ml of phage, and the cells were allowed to become confluent (4 to 6 days). In other experiments, confluent monolayer cultures were exposed to phages by replacing noncontaminated spent medium with fresh medium containing phages at a concentration of 1.1 × 10⁴ PFU/ml. The cells were then allowed to incubate with the virus for 4 additional days at 37 C. Triplicate cell cultures were grown in 25-cm² plastic flasks and used for each determination of phage by plaque assay. Each flask contained 6 ml of medium of which 0.1 ml was removed and tested in duplicate for phage content.

 Supernatant fluids from the various cultures were collected and stored at 4 C until assayed for phage. None of the fluids tested were concentrated prior to being assayed. Cell lysates were prepared from a 1% suspension of cells in 0.15 M NaCl by three freeze-thaw cycles. Media and cell lysates were assayed for phage by standard methods by using the C-3000 strain of E. coli (1, 2).

RESULTS AND DISCUSSION

After 4 days, medium from cell cultures infected at the time of their initiation contained about 50% of the inoculated phage. This was in contrast to medium from cultures infected as monolayers in which no decrease in phage titer was observed (Table 1). Entrapment or adsorption of phages by cells which failed to adhere to the vessel surface might account for part of the decreased recovery because the unattached cells were removed from the medium by low-speed centrifugation before plaque assay, and therefore their phage content was not determined.
Another factor that may lead to the decreased recovery of phages is the generally higher phagocytic and metabolic activity of cells in the logarithmic phase compared with those in the stationary phase of cell growth.

Table 2 presents data on the survival of phages introduced into monolayer cell cultures and into complete tissue culture medium without cells. The data indicate that the φV-1L is able to persist at 37 C for at least 4 days with no apparent loss in titer either in a cell culture environment or in tissue culture medium alone.

Table 3 shows that daily medium changes left four to 10 times the amount of phage that would be expected on the basis of serial dilutions of a residual volume (0.5 ml) of medium at each change. The five successive medium changes in this experiment reduced the inoculated phage concentration by a factor of 3.7 x 10⁴. The total effect of the medium changes is equivalent to the manufacturing process of washing tissue culture monolayers to reduce the concentration of animal serum in the final virus vaccine to a minimum dilution of 1:1,000,000 (6). In the experiment reported here, the concentration of phage in the final medium was still 25 PFU/ml.

In other experiments, phage could be recovered from the media for 12 days when diploid cell lines WI-38 and DBS-FRhL-2 were inoculated with 4.7 x 10⁷ and 5.5 x 10⁸ PFU, respectively, and medium was changed every 3 to 4 days. These findings suggest that some phages may be reversibly adsorbed to the surface of cells and that a new steady equilibrium is established after each medium change which releases more phages from the cells into the medium.

Recoverability of phages from several cell culture systems during certain operations usually employed during the life of a culture is shown in Table 4. The reduction in phage titer found at the first medium change (day 6) was consistent with that shown for cultures infected upon initiation (Table 1). When the contamination was not reintroduced, some of the bacterial viruses were removed during such operations as medium changes and trypsinization. When the input multiplicity of virus was high (~20 PFU/cell), virus could be easily detected in the cell lysate fraction. The amount of virus recovered from the various cell culture systems was remarkably uniform. Similar results have been obtained when CE cells were inoculated with bacteriophage T₂.

The results of the study reported here attest to the persistence of phages in cell cultures once introduced, and the data are consistent with the suggestion that bacterial viruses can be carried

**Table 2. Recovery of φV-1L phages from tissue culture medium alone and from monolayer culture fluids of primary chicken embryo held at 37 C without change of medium**

| Day | Medium only (PFU/ml) | Cell cultures (PFU/ml) |
|-----|---------------------|------------------------|
| 0   | 1.1 x 10⁶           | 1.1 x 10⁶              |
| 1   | 1.0 x 10⁴           | 1.3 x 10⁴              |
| 2   | 1.0 x 10⁴           | 1.3 x 10⁴              |
| 3   | 1.0 x 10⁴           | 1.1 x 10⁴              |
| 4   | 1.0 x 10⁴           | 1.1 x 10⁴              |

*Samples of 0.2 ml were taken each day from the same three containers.

**Table 3. Recovery of φV-1L phages from monolayer culture fluids of primary chicken embryo after daily medium changes**

| No. of medium changes | Actual* PFU/ml remaining | Theoretical* PFU/ml remaining | Calculated* PFU/ml removed | % Phage removed (%) |
|-----------------------|--------------------------|-----------------------------|---------------------------|---------------------|
| 0                     | 1.1 x 10⁶               | 1.1 x 10⁶                   | 6.2 x 10⁴                 | 56                  |
| 1                     | 4.8 x 10⁴               | 8.5 x 10⁴                   | 4.1 x 10⁴                 | 85                  |
| 2                     | 7.0 x 10⁴               | 6.5 x 10⁴                   | 6.5 x 10⁴                 | 93                  |
| 3                     | 5.0 x 10³               | 5.0 x 10³                   | 4.8 x 10⁴                 | 97                  |
| 4                     | 1.5 x 10²               | 3.9 x 10³                   | 3.2 x 10³                 | 83                  |

*Fluids taken from three monolayer cultures per day, pooled, and assayed.
*Assuming 0.5-ml residual medium at each 6-ml medium change and a serial reduction in concentration by a factor of 0.5/6.5, or 92.3%.
*Difference in actual concentration remaining between 2 consecutive days and after one medium change.
*Calculated amount removed divided by the actual amount present on the previous day times 100.
*Represents initial concentration before medium changes.
TABLE 4. Recovery of \(\phi V-1\) phages from cultures of diploid cell lines and primary cells after a single exposure to virus at the time of their initiation

| Cell cultures | Preinfected cell lysate (PFU) | Day 0 | Day 6 | Day 7 |
|---------------|-------------------------------|-------|-------|-------|
|               | Phage inoculum* (PFU)         | Medium (PFU) | Medium before trypsinization (PFU) | Used trypsin solution (PFU) | Postinfected cell lysate (PFU) |
| WI-38         | 0                             | 1.2 \(\times\) 10^8 | 4.6 \(\times\) 10^4 | 2.7 \(\times\) 10^3 | ND | 0 |
| DBS-FRhL-2    | 0                             | 1.2 \(\times\) 10^8 | 4.5 \(\times\) 10^4 | 2.8 \(\times\) 10^3 | ND | 0 |
| 1° VMK        | 0                             | 1.2 \(\times\) 10^8 | 4.5 \(\times\) 10^4 | 2.6 \(\times\) 10^3 | ND | 0 |
| 1° CE         | 0                             | 1.2 \(\times\) 10^8 | 4.6 \(\times\) 10^4 | 2.5 \(\times\) 10^3 | ND | 0 |
| 1° CE         | 0                             | 6.6 \(\times\) 10^8 | ND | 6.2 \(\times\) 10^6 | 8.0 \(\times\) 10^4 | 4.5 \(\times\) 10^4 |

* In 6-ml volume of medium containing 2.0 \(\times\) 10^4 PFU/ml.
† ND, Not determined.
‡ 1° VMK, Primary vervet monkey kidney.
§ 1° CE, Primary chicken embryo.

over to vaccines produced in contaminated substrates.

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