surviving 13 days) gave higher titers and more reproducible results than the egg lethal dose. Strain differences in egg pathogenicity were demonstrated. It took 5 infectious doses of the Bour and TW-3 strains to produce one lethal dose, while 80 infectious doses of the TW-1 strain equalled one lethal dose.

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Persistent Infection of Human Cells (HeLa) with Adenovirus Type 12.* (28979)

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The oncogenic effect of human adenovirus type 12 (A-12) in newborn Syrian hamsters has been clearly established(1). Transformation experiments(2) with explants of primary human tissues were performed to determine the effect of A-12 on human tissues. It was found that few, if any, cells survived the cytopathic effects of the virus for extended periods. Although one of the major reasons for testing the oncogenic properties of the adenoviruses was their classical latency in human tissues(3), type 12 has been isolated only from fecal material and its possible role as a latent virus in human tissues has not been previously established.

To determine if methods could be found to prevent the extensive cytopathic effects of A-12 and to determine if a latent infection in human tissues could be established, a HeLa cell model system was employed. Particular attention was given to the serum components of the medium since Ginsberg reported that the establishment of carrier cultures with types 3 and 4 adenoviruses was dependent on an inhibitor fraction in human sera(4).

Materials and methods. Adenovirus type 12. Human adenovirus type 12, strain Huie, was obtained from American Type Culture Collection. The stock virus used was of the 8-10th passage in HeLa cells in this laboratory, and had a titer of approximately 2.5 $\log_{10}$ per 0.1 ml as determined by cytopathic effect on HeLa cells, with final reading on day 5.

Medium. Eagle's medium was used, with different sera and concentrations as described in the text. All sera were heat inactivated at 56°C for 30 minutes. Media contained 200

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units of penicillin, 200 mg of streptomycin, and 20 units of Mycostatin per ml.

HeLa cultures. HeLa cells, originally derived from a carcinoma of the uterus, were obtained from Microbiological Associates. Uninfected HeLa cells were routinely grown in Eagle's medium with 10% calf serum; carrier HeLa cells were routinely grown in Eagle's medium with 20% normal human serum, unless indicated otherwise in the text. Cultures were incubated at 37°C and subcultured by trypsinization every 4-7 days. When the medium was changed to a different concentration or type of serum, the monolayers were washed 3 times with a balanced salt solution before addition of the new medium.

Titrations. All titrations were done in HeLa tubes seeded with 60,000 cells per ml in Eagle's medium with 10% agamma calf serum.† The inoculum was 0.1 ml of virus in 10-fold dilutions, using 1 to 4 tubes per dilution. All titers are expressed per 0.1 ml.

Cloning. Sixty millimeter petri dishes were seeded with 100 cells. The dishes were examined microscopically for clones, and after 8-14 days were stained with Giemsa and counted.

Cell growth curve. Two-ounce prescription bottles were seeded with a known number of control or infected cells. On days 3, 5, and 7, bottles were trypsinized; cell volumes were measured in haematocrit tubes and cell counts were made with a hemocytometer.

Hyperimmune rabbit serum (HIRS). Rabbits were inoculated at weekly intervals with A-12 by the intraperitoneal route. The serum obtained after 4 inoculations had a titer of 1:512.

Results. Effects of different serum components on the titer of A-12. To test the effect of the serum component of the media on the titer of A-12, HeLa tubes grown and maintained in Eagle's medium containing 10% calf serum, 10% agamma calf serum, 10% horse serum, or 20% human serum, were inoculated with 10-fold dilutions of stock virus and observed for characteristic adenovirus cytopathic effect. A delay in CPE and a reduction in final titer was observed with horse serum, calf serum, and human serum as compared to agamma calf serum (Fig. 1).

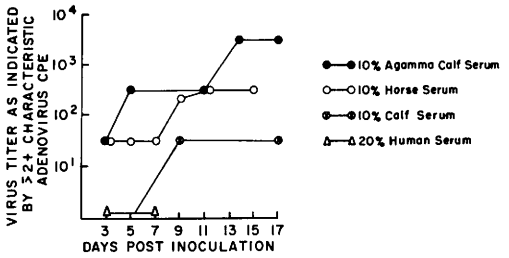


FIG. 1. Effect of serum component of media on titer of A-12 in HeLa cells.

Establishment of carrier cultures and general characteristics. It appeared that human serum was at least as effective as any other serum in suppressing the cytopathic effects of A-12; therefore, bottles containing a complete monolayer of HeLa cells in either 10, 20 or 40% human serum or 10% horse serum were inoculated with 200 $TCID_{50}$ of A-12. On the fourth day post inoculation, subcultures of the bottles with human serum were made. At this time bottles containing horse serum showed extensive degeneration while bottles with human serum were similar to uninfected controls. Cultures in this experiment (Exp. 21) were carried through 6 subcultivations with each concentration of human serum. The 20% human serum concentration appeared optimum for the carrier culture system, since cells with 10% human serum showed relatively more CPE after the first subculture, while the cells with 40% human serum did not grow as satisfactorily. Low titers of virus were found at all passage levels tested except in the first passage with 40% human serum where no virus was found, and at the fifth passage with 10% human serum where 3.5 logs of virus were found.

Two new series were initiated in medium containing 20% human serum only and an inoculum of 15 $TCID_{50}$ (Exp. 39 and 87). In Exp. 39, infected cultures were carried through 16 subcultivations and cultures in Exp. 87 are now in their 13th subcultivation. With each passage series it was observed that

† Newborn calf serum specially processed for removal of gamma globulin, Hyland Laboratories, Los Angeles, Calif.

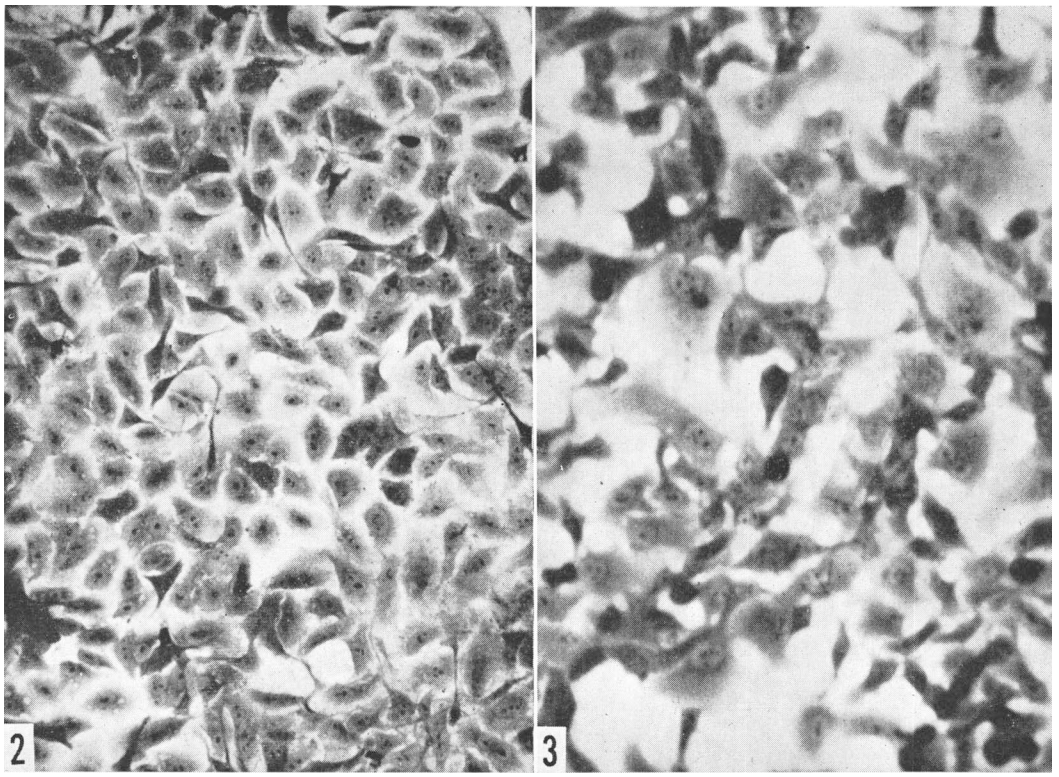


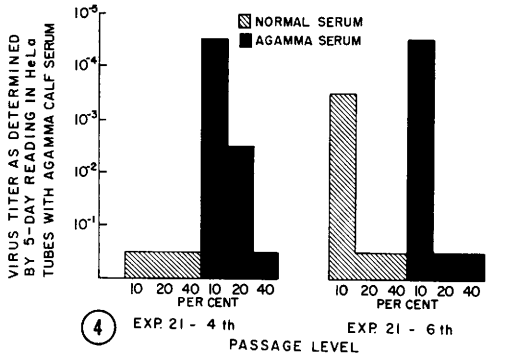
FIG. 2. Uninfected HeLa cells.
FIG. 3. Carrier HeLa cells. Giemsa stained cover slip, third passage level, 3 days since subculture.

infected cells grew best when subcultured every 3-4 days as usually an increased CPE was observed after this time. In each series, more extensive CPE was observed during the third to fifth subcultures than in earlier or subsequent passages. Giemsa stained cover slips of normal and carrier cells are shown in Fig. 2 and 3.

Effect of different concentrations of human serum and chicken serum with tryptose phosphate broth on carrier cultures. In Exp. 39, 7th passage level, cultures were changed to media with the following components: 2.5, 5.0, 7.5, 10, 15, and 20% human serum, and 7.5% chicken serum with 25% tryptose phosphate broth. No significant differences were noted in CPE after 5 days of observation; only trace amounts of virus were detectable in any of the cultures.

Comparison of agamma human serum and normal human serum. In an effort to determine the effects of antibodies or serum inhibitors, presumably in the gamma globulin fraction, agamma human serum was employed. In Exp. 21, at the 4th and 6th passage levels, cultures were changed to media containing 10, 20, or 40% agamma human serum while controls were carried on the same concentrations of normal human serum. At the 4th passage level cells with 10 or 20% agamma human serum had a 2+ CPE while controls had a 1+ CPE. At the 6th passage level no differences in CPE were observed.

After 3 days' observation, all cultures were frozen and thawed 3 times and the fluids were assayed for virus. At both passage levels, virus titers in fluids from cultures from each concentration of normal human serum were $10^{0.5}$, except at the 6th passage level with 10% normal human serum where the titer was $10^{3.5}$ (Fig. 4). In comparison with the respective concentrations of normal human serum, with 10% agamma human serum 4.0 logs more virus were found at the 4th



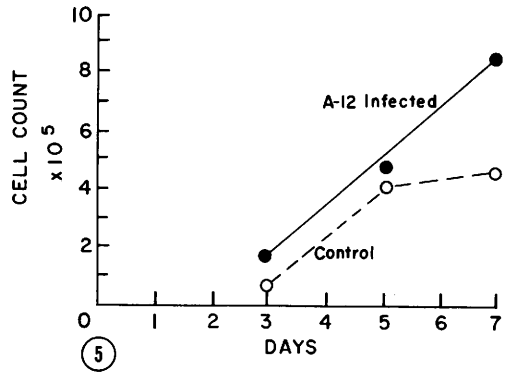


FIG. 4. Effects of varying concentrations of normal and agamma human serum on virus titer.

FIG. 5. Growth rate of A-12 infected and uninfected HeLa cells.

passage level and 1.0 log more virus at the 6th passage level; with 20% agamma human serum 2 logs more virus were found at the 4th passage level and at the 6th passage there was no difference; with 40% agamma human serum no difference was found (Fig. 4).

Growth rate of carrier and uninfected cells. A growth curve of the infected cells was made by taking cell counts on days 3, 5, and 7 (Fig. 5). The growth of infected and uninfected cells for the first 5 days appeared to be the same, but after day 5 the infected cells remained in log phase while the uninfected cells went into a stationary phase.

Estimation of number of virus yielding and infected cells. To determine the number of virus yielding cells, suspensions were prepared containing approximately 10 cells per ml. After freezing and thawing 3 times, aliquots of each of the 10 suspensions were inoculated into HeLa tubes. These were observed for 14 days and subcultured for an additional 5 days. Only 3 groups of cells yielding virus were found.

Estimation of the number of infected cells was made by examining cultures on cover slips which had been stained with May-Grünwald-Giemsa for adenovirus cellular changes. Forty-eight hours following seeding, 21% of the cells examined showed evidence of cytopathic changes, while at 72 hours the percentage of cells with evidence of infection approximately doubled.

Cloning. Cloning experiments with both normal and infected HeLa cells were made. Eight to fourteen days after seeding, approximately 30% of the normal HeLa cells had established clones, while no clones were observed with infected cells.

Sensitivity to super-infection. Carrier HeLa cells were subcultured in tubes and superinfected with 320, 32, or 3.2 $TCID_{50}$ of A-12. Cytopathic effects were no greater than those observed in carrier cells without superinfection which involved 50% of the cell sheet after 5 days. For comparison, normal HeLa cells grown and maintained in 10% agamma calf serum and receiving the same viral inoculum showed extensive degeneration between 2 and 5 days post inoculation, depending on virus dosage.

Effects of hyper-immune rabbit serum. Hyperimmune rabbit serum was added to the culture medium of carrier cells for 5 consecutive passages. Results were inconclusive because of the cytotoxicity of the hyperimmune rabbit serum and because of the low levels of virus in companion cultures without the immune sera, but virus was detected in cultures with HIRS at some passages.

Chromosomal changes. Dr. Audrey Fjelde kindly examined the chromosomes of a total of 55 cells of carrier cultures at 2 different passage levels. Four cells with polyploidy and 2 or 3 chromosomal aberrations (2 breaks, 1 constriction) were observed.

Discussion. Inhibition of type 12 adenovirus by various sera is consistent with reports by others of similar effects against other types of adenoviruses(5). Ginsberg found that an adenovirus inhibitor in calf

serum had many of the properties of antibody, except specificity(5). It has not been determined whether the inhibition by human serum is related to inhibitor, antibody or both, but the effects of either would in all probability be similar if not the same, *i.e.*, interference with virus adsorption.

In the initial experiments, it appeared that the establishment of a carrier state was dependent on the human serum, since parallel cultures maintained with horse serum had extensive degeneration. In subsequent experiments to demonstrate this dependence with a) lower concentrations of human serum, b) chicken serum and tryptose phosphate broth, and c) agamma human and calf serum, extensive CPE was not observed although with 10 or 20% agamma human serum, higher titers of virus were found. In these respects, the A-12 carrier HeLa cells appear to be different from the adenovirus type 3 system described by Ginsberg as extensive degeneration was observed when the inhibitory human serum was replaced with inhibitor free serum(4). Since mechanisms postulated or demonstrated for other virus-carrier cell culture systems include selection of resistant cells, interference by non-infectious virus and interferon(6,7), experiments to define the role of these factors in the A-12 system are indicated.

Reasons for the enhanced growth rate of carrier cells between days 5 and 7, as compared to uninfected cells are not apparent. Similar cell counts were not made with each series of carrier cells established. It is of interest that one characteristic of primary hamster cells infected and transformed by polyoma virus is an enhanced growth rate(2). It is difficult to compare the chromosomal changes with the findings of others because of the nature of the cells employed.

The differences obtained between virus yielding cells and infected cells may be related to the relative insensitivity of HeLa cells for the detection of virus when compared with cultures of primary embryonic kidney. The relatively large number of infected cells may be the explanation for the failure to obtain clones.

Summary. The establishment of an adenovirus type 12 cell carrier system is described. Characteristics of the carrier cultures include an initial dependence on inhibitor or antibody in human serum, an enhanced rate of growth after the fifth day as compared to uninfected cells, a relatively high percentage of infected cells, but a low percentage of detectable virus yielding cells, and relative resistance to super-infection. Secondary mechanisms responsible for the carrier state may include the selection of resistant cells, interference by non-infectious virus and interferon.

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