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# Feline Herpesvirus Infection in Fused Cultures of Naturally Resistant Human Cells

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Feline herpesvirus (FHV) attaches to, but does not penetrate, naturally resistant human embryonic lung cells. When the cells with attached virus are subsequently fused with inactivated Sendai virus, FHV causes characteristic cytopathic effects, but no infectious virus can be recovered from the infected cells.

Replication of virus can take place in naturally resistant cells, provided that measures are taken to circumvent the barrier to infection presented at the cell surface. Holland and his associates (9) initiated infection in resistant chick embryo cells with poliovirus ribonucleic acid (RNA). Subsequently Enders and his co-workers (4) and Neff and Enders (11) showed that complete poliovirus replicates in hamster cells and chick embryo cells exposed to inactivated Sendai virus. It was suggested that the virus was incorporated into the cytoplasm of these naturally resistant cells during the Sendai virus-induced process of cell fusion in which the cellular membrane is impaired or ablated. It was proposed that this technique might be employed as a means of isolating agents which have not yet been cultivated in vitro.

The foregoing observations raised the question of whether the natural barrier in cells resistant to infection by deoxyribonucleic acid (DNA) viruses, which replicate in the nucleus rather than in the cytoplasm, could also be overcome by cell fusion. For it seemed that with such intact agents the nuclear membrane might provide a second barrier to successful entry of the virus into the only cellular compartment in which replication can take place. An answer to this question might not only extend the potential usefulness of Sendai virus in overcoming cellular resistance but also help to define other barriers to infection that may exist in the naturally insusceptible cell.

Feline herpesvirus (FHV; 3) was selected as the test organism, since this agent replicates in vitro to high titers and produces easily recognizable intranuclear inclusion bodies in feline kidney (FK) cells but has been found to exhibit no detectable effect on cells from a variety of other species (2, 10).

## MATERIALS AND METHODS

**Viruses.** FHV, isolate number 1217, was generously supplied by Abraham Karpas (10). Pools of virus were prepared by harvesting infected primary feline kidney cells and medium after the development of extensive cytopathic effect. The virus suspension was frozen and thawed three times, clarified by slow centrifugation, and stored at −60 C. Stock preparations consisting of materials representing the 4th to 13th virus passages were employed. Infectivity titers of these preparations in feline kidney cell monolayer cultures varied from log 6.5 to 7.5 $TID_{50}$/0.1 ml. Sendai virus was prepared and inactivated with β-propriolactone (BPL) and stored as described by Neff and Enders (11). No residual infectious virus was detected in any of the preparations.

**Cells.** (i) To prepare FK cells, kidneys from immature cats were trypsinized and grown in prescription bottles, tubes, or plastic dishes with Eagle's medium containing 10% fetal calf serum. After the monolayers were complete, the serum concentration of the medium was decreased to 5%. (ii) To prepare human embryo lung (HEL) cells, lungs from an aborted fetus were trypsinized, grown in Eagle's medium enriched with 10% fetal calf serum, and maintained with the same medium containing 2% serum. Cells from the 5th to 12th subcultures of this cell line were used. (iii) FK and HEL cells were cocultivated by adding trypsinized FK cells to incomplete monolayers of HEL cells.

**Ultraviolet inactivation of FHV.** A 2-ml amount of virus pool in a 35-mm petri dish was exposed for 5 min to ultraviolet light from a General Electric 30-w germicidal tube at a distance of 5 cm.

**Trypsin inactivation of FHV.** Gresser and Enders (7) showed that trypsin rapidly inactivates herpes simplex virus. Accordingly, trypsin was added to remove extracellular FHV from cell cultures after exposure to high concentrations of the agent. The infected cultures were exposed to 0.25% trypsin for 45 min at 37 C, sedimented without centrifugation, and resuspended in medium.

**Virus assay.** FHV infectivity titers were determined by the standard end-point dilution technique in tubes and by plaque counts on monolayer cultures. These methods gave similar results if primary FK cells were used. For plaque assay, 0.2 ml of virus suspension was added to 35-mm plastic petri dishes for 2 hr and then overlaid with 3 ml of Earle's balanced salt solution containing 0.9% agar, 0.5% lactalbumin hydrolysate, and 10% fetal calf serum. Plaques were grossly visible in 2 to 3 days and increased in number until 4 to 5 days after inoculation. Unless otherwise noted, all titrations were made after cultures had been frozen and thawed three times.

**Fixing and staining.** Cultures were fixed in Bouin's fluid, imbedded in collodion, and stained with hematoxylin and eosin (5).

**Inoculation and fusion of cells.** Monolayer cultures of cells in 150-mm tubes were inoculated with FHV suspended in Eagle's medium containing 2% fetal calf serum. An 0.1-ml amount of inactivated Sendai virus in appropriate concentration (11) was added after 30 min. Cultures were incubated at 36 C in a roller wheel for 6 hr. Inoculated cultures were then either washed seven times with 5 ml of Hanks' solution to remove unadsorbed virus or were washed two times and exposed to trypsin to inactivate extracellular virus.

## RESULTS

**FHV infection of FK cells.** Inoculation of FK cells with varying doses of FHV resulted in complete cytopathic effect (CPE) in 1 to 7 days, depending on the size of the virus inoculum. As far as it was possible to determine by inspection, all cells developed intranuclear inclusion bodies. Fusion of cells induced by FHV was extensive when the medium contained 10% serum. After inoculation of FHV at a multiplicity of 3 plaque forming units (PFU) per cell, replication of infectious virus began in 6 to 8 hr and increased rapidly thereafter until an average yield of 200 infectious particles was produced by each cell (Fig. 1). It was also determined that the addition of inactivated Sendai virus did not affect the yield of FHV by FK cells.

**FHV inoculation of HEL cells.** Inoculation of HEL cells with FHV at multiplicities of 200 PFU per cell consistently failed to cause any detectable CPE. Mitosis continued at the same rate as in control cultures, and cells from exposed cultures were easily subcultured.

Co-cultivation of FK and HEL cells resulted in the formation of sheets of fibroblastic cells containing distinct islands of epithelial cells. Infection of these cultures converted the islands of FK cells into large syncitial cells with nuclei containing intranuclear inclusions. The surrounding human fibroblasts did not appear to be affected by this close contact with the infected feline cells (Fig. 2). On further cultivation, the giant cells detached from glass and disappeared.

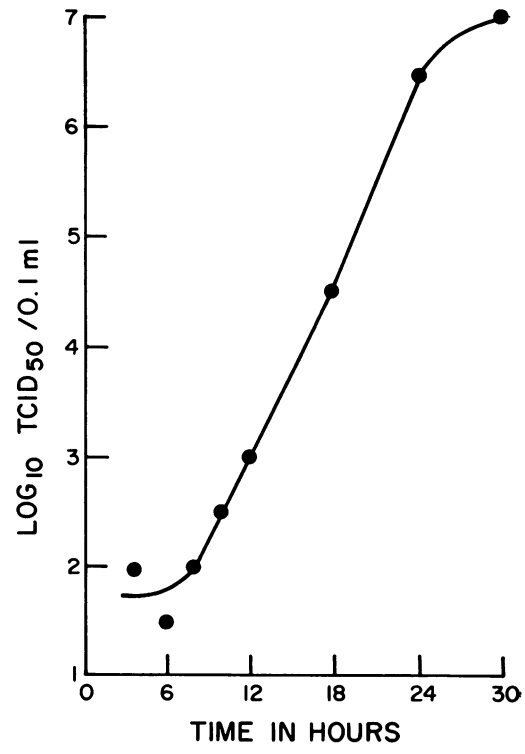


FIG. 1. *Total virus recovered from primary FK monolayer cultures at intervals after inoculation of FHV (input multiplicity = 3).*

In contrast, the fibroblasts remained viable for at least 2 weeks and formed monolayers on the surfaces from which the giant cells detached. Although the fusion of a few human fibroblasts into the giant cells cannot be excluded, it would seem there was no alteration in the cytopathogenicity of FHV for human cells during replication of the virus in mixed cultures.

**Attachment of virus.** Experiments to quantitate virus adsorption to both kinds of cells were carried out in plastic dishes containing monolayers of either HEL or FK cells. The cells were exposed at 25 C to approximately 100 PFU of FHV in 0.4 ml of medium. At intervals thereafter, the fluid in individual plates was aspirated and titered for unadsorbed virus. Virus disappeared from the fluid covering FK and HEL cells at approximately the same rate. Little virus was lost by nonspecific adsorption to plastic or by thermal inactivation in plastic dishes containing no cells (Fig. 3). Large quantities of FHV were also directly shown to be closely associated with HEL cells. Tube cultures of such cells, which were exposed to log 7.0 $TID_{50}$/0.1 ml of FHV and after 6 hr were washed seven times with 5-ml portions of Hanks' solution, were found to contain 6.0

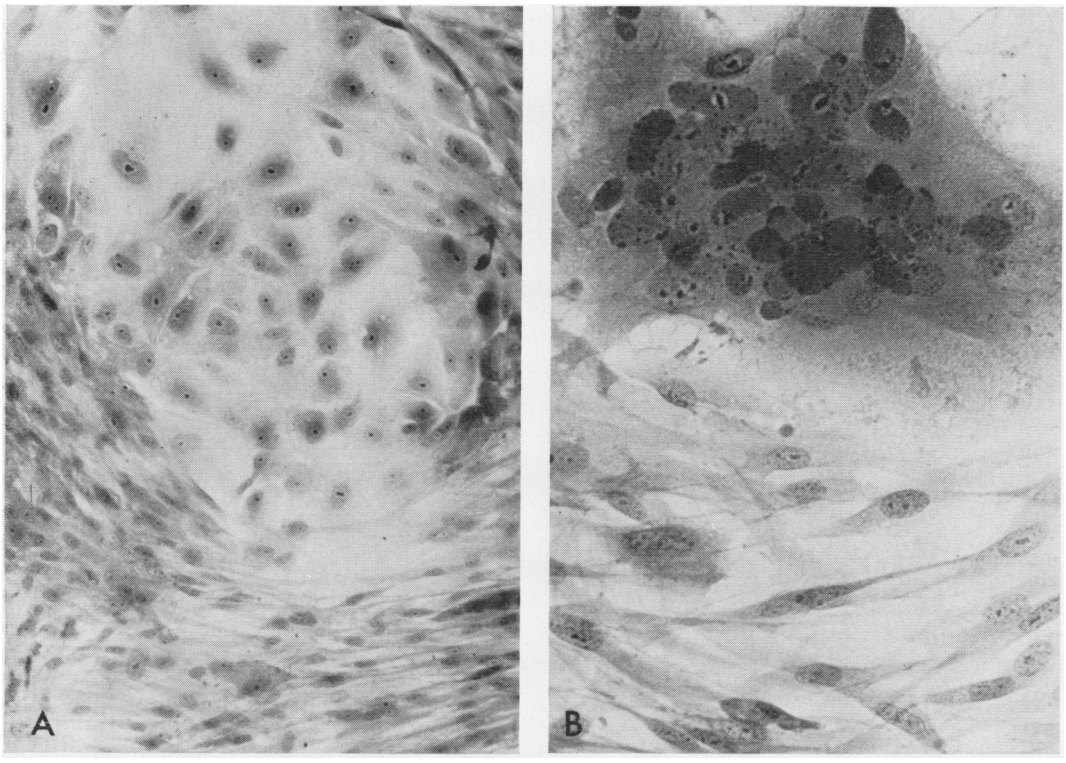


FIG. 2. (A) *Mixed cultures of human embryonic fibroblasts and islands of epithelial cells presumably of feline origin. H & E stain. ×160.* (B) *Mixed cultures of human embryonic fibroblasts and FK cells inoculated with FHV (input multiplicity = 200 PFU/cell). Islands of epithelial cells have formed giant cells in which nuclei contain inclusions characteristic of those formed in feline cells. These inclusions typically do not present distinct halos. The adjacent fibroblasts appear unaffected. Hematoxylin and eosin stain. × 320.*

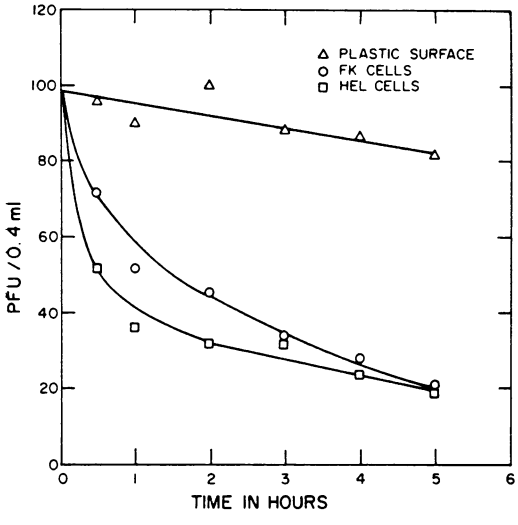


FIG. 3. *Adsorption of FHV by monolayer plate cultures of HEL and FK cells. Virus disappeared from each inoculum at approximately the same rate. Plates without cells indicate loss of virus by nonspecific attachment and heat lability.*

$TID_{50}$/0.1 ml (Fig. 4) when the entire cultures were frozen and thawed. The supernatant fluids from these cultures contained log 3.5 $TID_{50}$/0.1 ml prior to freezing and thawing.

**Penetration of virus.** The penetration of FHV into HEL and FK cells was determined by quantitating the disappearance of attached virus after exposure for 2 hr to cell monolayers at 25 and 4 C. FHV disappeared from FK cultures 100 times faster at 25 than at 4 C. FHV adsorbed to HEL cells disappeared at the same rate at 4 and 25 C. The rate of disappearance at both these temperatures was the same as that at 4 C in FK cells. In other experiments, trypsin was found to inactivate extracellular FHV very rapidly (Fig. 5). When HEL cells that had adsorbed large quantities of FHV were exposed to the same concentration of trypsin, the cell-associated virus was inactivated at the same rate as extracellular virus (Fig. 4).

**FHV infection of HEL cells exposed to BPL-inactivated Sendai virus.** Polykaryocytes resulting from the fusion of noninfected HEL cells with

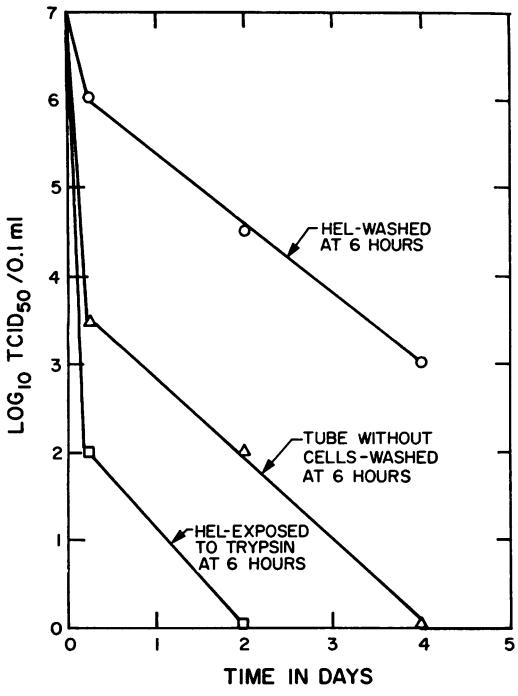


FIG. 4. *Effect of washing and trypsinization on FHV adsorbed to HEL cells. Repeated washings 6 hr after inoculation failed to remove large quantities of virus from tubes containing HEL cells (upper curve). As a control, repeated washing of tubes without cells to which the same amount of virus was added resulted in the removal of a large proportion of the virus (middle curve). Subsequent treatment of inoculated and washed HEL cultures with 0.25% trypsin for 45 min inactivated most of the cell-associated virus (lower curve).*

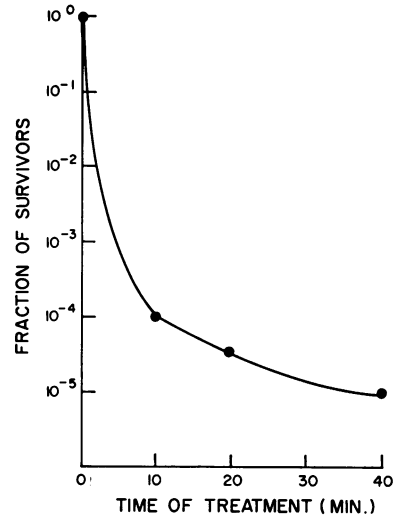


FIG. 5. *Inactivation of extracellular FHV by 0.25% trypsin.*

inactivated Sendai virus remained viable for at least 2 weeks, as judged by morphological appearance. Fusion was apparent as early as 6 hr and complete at about 24 hr after exposure to Sendai virus (Fig. 6). When FHV in high multiplicities was added to the monolayer prior to fusion, characteristic CPE was subsequently noted (Fig. 7). Early changes consisted of retraction and increased granularity of giant cells with disruption of the cell sheet. This process was evident on the first day after inoculation and continued for 2 to 3 days, until only a few unfused cells remained adhered to the glass. On continuing incubation, these remaining cells eventually multiplied and recovered. The nuclei in single polykaryons varied in appearance. Some nuclei exhibited no observable changes, whereas others showed progressive CPE (Fig. 8), distinguished by fragmentation of the chromatin which was followed by inclusion body formation and finally extreme karyorrhexis. The number of infected polykaryons and the number of nuclei with inclusions in each polykaryon were roughly proportional to the size of the inoculum, with doses ranging from log 5.0 to 7.5 $TID_{50}$/0.1 ml of virus, and did not increase after the second day of infection. With smaller amounts of virus, cytopathic changes were evident only in scattered areas. With the larger quantities of virus, as many as 40 to 50% of nuclei might contain inclusions. No mononuclear cells were found that contained intranuclear inclusions. That such CPE was the result of FHV infection was apparent from the finding that the ultraviolet-irradiated FHV induced no changes in fused cultures of HEL cells.

Infected, fused cultures of HEL cells exhibiting extensive CPE were found to contain, after 2 days, little or no infectious virus when the input virus was inactivated by trypsin at 6 hr after inoculation (Fig. 9). Attempts were made to recover infectious virus which may have been only partially defective from fused HEL cell cultures. Infected HEL cells presenting CPE were directly transferred without freezing and thawing to FK and HEL cultures. Some of the FK cultures were then also exposed to inactivated Sendai virus concentrate. No increase in virus yield in FK cells was obtained. CPE was not observed after material from infected HEL cultures was serially transferred in this system.

## DISCUSSION

The results show that FHV, when added to naturally resistant HEL cells, induced infection characterized by inclusion body formation and

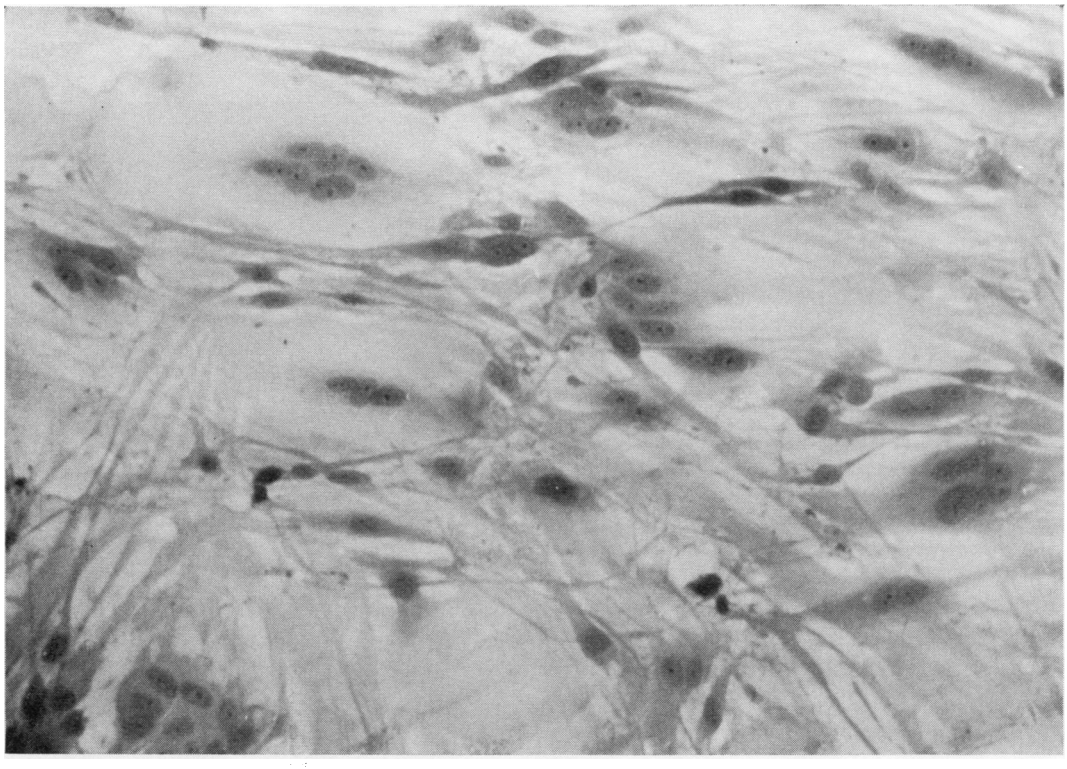

FIG. 6. *HEL cells 48 hr after addition of Sendai virus. Hematoxylin and eosin stain.* × *270.*

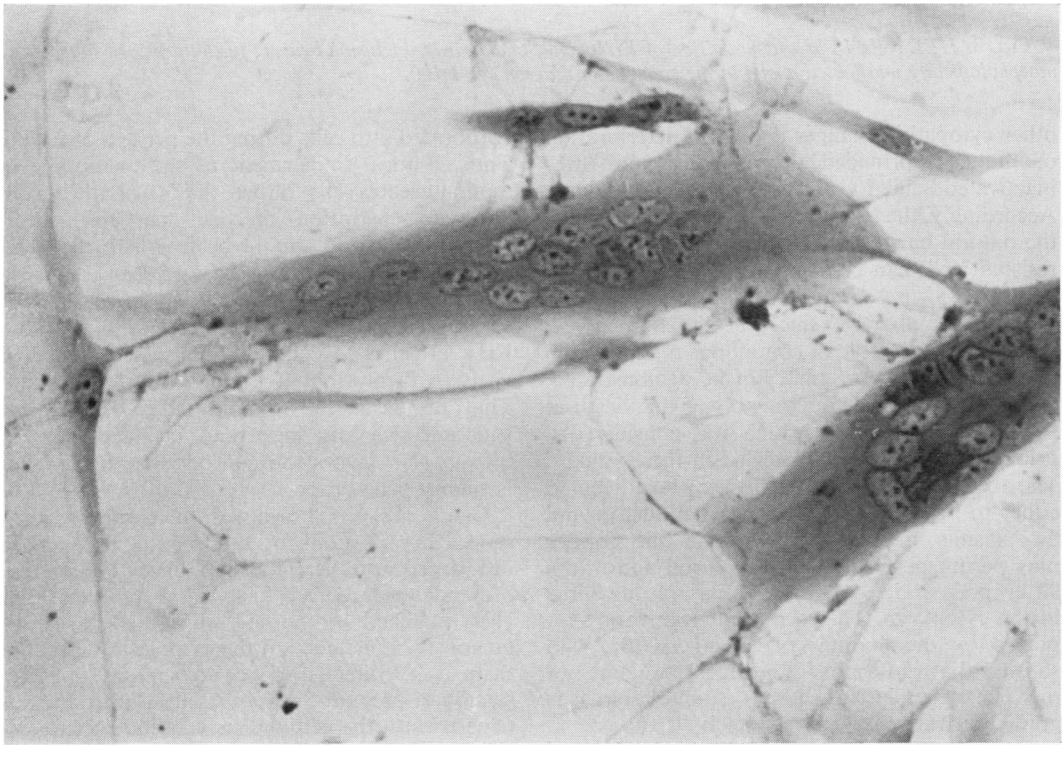

FIG. 7. *HEL cells 48 hr after addition of FHV and Sendai virus. Many nuclei in polykaryons contain inclusions. Mononucleate cells are normal in appearance. Hematoxylin and eosin stain.* × *270.*

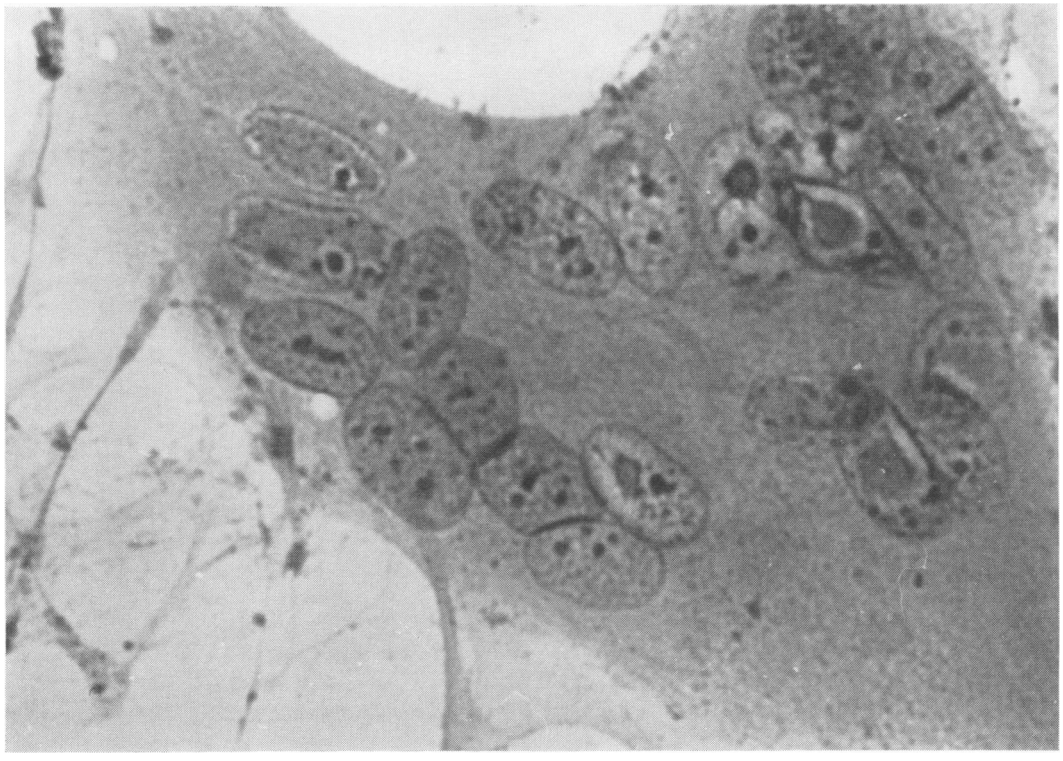

FIG. 8. *HEL cells 48 hr after addition of FHV and Sendai virus. Inclusion-bearing nuclei are seen adjacent to morphologically unaffected nuclei in the same heterokayron.* × *1,400.*

other cytopathic changes ending in deterioration of the cell, provided that concentrated BPL-inactivated Sendai virus was subsequently added. Accordingly, the technique of cell fusion by which the natural barrier to entry of RNA viruses into resistant cells can be overcome is also applicable to DNA viruses. The infection thus induced, however, is abortive since, in the human cell system, virus capable of continuous replication in susceptible FK cells could not be demonstrated.

No additional barrier to entry of the virion at the nuclear membrane which was considered a priori a possibility was revealed in these studies. The absence of a second barrier may be attributed either to the differences between the nuclear and cytoplasmic membranes which in the former may permit penetration of the virion and in the latter prevent it, or to uncoating of the virus in the cytoplasm. The second alternative is supported by the findings of Hochberg (8), who showed that radioactively labeled DNA but not the coat proteins of herpes simplex virus is gradually transported into nuclei.

The present results accord with the concept of Enders et al. (4) that virus is mechanically incorporated into cells during the process of fusion. Thus, if focal impairment of the membrane of mononucleate cells rather than fusion of cells allowed penetration of virus particles, intranuclear inclusions should be found in mononucleate cells. None, however, were seen. It is also unlikely that Sendai virus acts by increasing adsorption of virus to resistant cells, because FHV attaches to cells in the absence of Sendai virus so firmly that it is not easily washed off. That the virus thus bound to cells does not spontaneously disappear and is inactivated by trypsin also favors the concept that the barrier is against penetration.

Okada (12) has shown with the electron microscope that portions of the cell membrane are introduced into the cytoplasm during the process of Sendai-induced cell fusion. It is probable that virus attached to the cell surface could simultaneously be included in the cytoplasm, although there is no direct evidence to support the suggestion at present. Such an artificial introduction of virus into the cell and its subsequent successful uncoating and entry into the nucleus would raise questions concerning the function of the

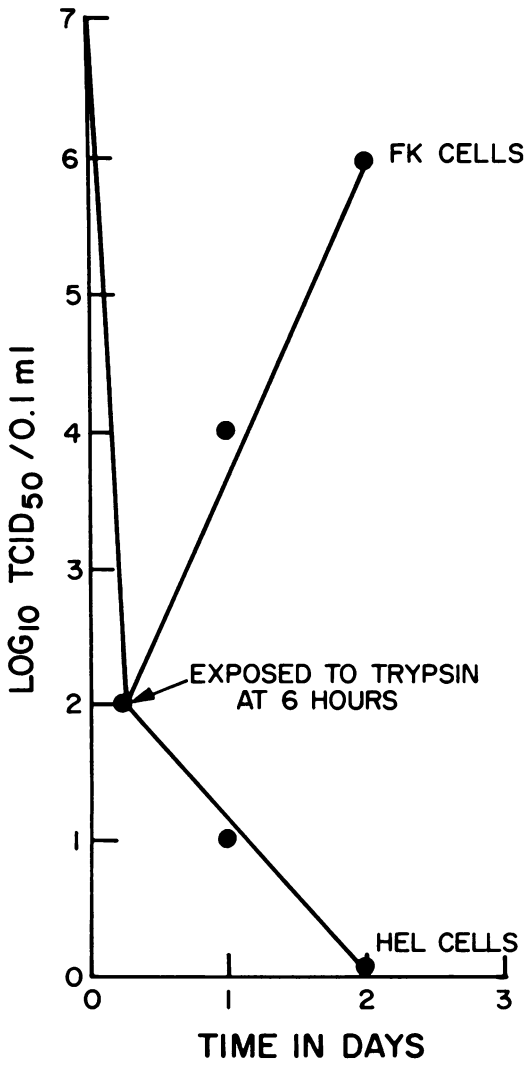


FIG. 9. *Total virus recovered from primary FK monolayers and human embryonic fibroblasts at intervals after inoculation of FHV (input multiplicity = 200) and subsequent exposure to inactivated Sendai virus. After 2 days, no virus could be recovered from HEL cells undergoing extensive cytopathic changes.*

steps usually involved in the entry of herpesvirus into cells. Epstein et al. (6) demonstrated by means of the electron microscope that, after attachment, herpes simplex virus is ingested into membrane-lined cytoplasmic vesicles. The outer envelope disappears while the particles are still visible in the vesicles. The central portion of the virus is later found free in the cytoplasmic matrix. Our findings indicate that the viral envelope and protein coat can be removed when the normal process of pinocytosis is apparently avoided.

It is interesting that only some of the nuclei in single polykaryons demonstrated CPE. Cell fusion occurs simultaneously with, or at least very early after, infection. Irregularity of signs of infection in the nuclear population of the polykaryons suggests, therefore, that virus-coded products which are made in the cytoplasm can act fully only in nuclei which contain a viral genome or that some essential products are made directly in infected nuclei. This suggestion is consistent with the finding that the proportion of nuclei in polykaryons with CPE is dependent on the size of the virus inoculum and is therefore presumably not determined by the physiological state of the nuclei. Roizman, Spring, and Roane (14), using fluorescent antibodies, have shown that some herpes simplex viral antigens are segregated in the nucleus or in the cytoplasm and that, within the limits of detection, each antigen accumulates in one compartment only.

The absence of infectious virus in the infected human cells points to one or more defects in the virus-cell relationship that are independent of the barrier to penetration. The number of potential defects in abortive infection probably equals or exceeds the number of genes in the infecting virus. One of them may be, as Spring and her associates (13) have shown in the case of abortive herpes simplex virus infection of dog kidney cells, the failure of envelopment of the virus at the inner nuclear membrane. Definition of the defects, however, in the replication of the FHV in the human cell system we have employed must await further experimentation. Preliminary electron microscopic studies of our abortively infected cells indicate that intranuclear virus-associated alterations are present. More detailed studies of changes in the fine structure of these cells will be reported later.

ACKNOWLEDGMENTS

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