Chronic Rabies Virus Infection of Cell Cultures

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Exposure of both mammalian and reptilian cells in tissue culture to different strains of fixed rabies virus resulted in a carrier type of infection. No cytopathic effect was observed in either type of culture; infected cultures could be maintained by cell transfer for unlimited numbers of passages. A consistent pattern of cyclically rising and falling levels of viral infection was observed by fluorescent antibody staining techniques and by titration of released infectious virus. Resistance to superinfection by vesicular stomatitis virus and the production of an interferon-like substance by infected cells indicated that the maintenance of a carrier type of infection may be interferon-mediated. The degree of susceptibility of rabies-infected cells to immunolysis by antirabies antibody in the presence of complement was found to be correlated with the amount of virus maturation occurring by budding through the cell membrane and not with the presence of immunofluorescent antigen in the cytoplasm of infected cells.

Rabies virus infection is characterized by a prolonged incubation period; several weeks or months may elapse between exposure and the appearance of clinical symptoms. Very little is known about the manner in which the virus infection is maintained in the organism during this period. Once the signs of rabies virus infection appear, rabies antigen may be detected preferentially in cells of the central nervous system, but the pathological changes observed usually lack the cytolysis and inflammation which characterize most viral encephalitides. The mechanism of cell injury is not yet understood.

The study of rabies virus-infected cells in tissue culture may provide information on the mechanisms underlying both the prolonged maintenance of infection during the presymptomatic period and the eventual alteration of neurons that leads to fatal dysfunction. Exposure of mammalian cells in tissue culture to rabies virus results in a carrier type of infection (5, 20). No cytopathic effect is observed, and rabies virus-infected cultures can be maintained by cell transfer for unlimited numbers of passages. An investigation of the endosymbiotic relationship between rabies virus and rabbit endothelial cell cultures was reported (6). The present study (a preliminary communication on this subject was previously presented [T. J. Wiktor and H. Koprowski, Bacteriol. Proc., p. 166, 1967]) is an inquiry into chronic rabies virus infection in cell cultures of hamster and reptilian origin. Each of these systems is characterized by cyclical rising and falling titers of released virus. Evidence is presented that this virus growth pattern is mediated by interferon in the hamster cell systems.

MATERIALS AND METHODS

Virus strains. Tissue culture-adapted, Pasteur, Pitman Moore (PM), Flury high egg passage (HEP) (20), and standard challenge virus (CVS-11) (10) strains of rabies virus were propagated in BHK-21 cells (13) as previously described (9). The mouse brain-propagated CVS-26 strain was also used. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in BHK-21 cell cultures.

Tissue cultures. Mammalian cells of cell line BHK-21, embryonic hamster cell line Nil-2 (4), and human diploid cell strain WI-38 (7), were propagated at 36°C in Eagle basal medium in Earle balanced salt solution (BME) containing 10% heat-inactivated fetal calf serum. Cells of the reptilian cell lines VH2 and VSW; both derived from the snake Vipera russelli (H. F. Clark, M. M. Cohen, and P. D. Lunger, Abst. In Vitro, vol. 6, p. 376, 1971) and GL-1 from the lizard Gekko gecko (3) were propagated in the same medium at 30°C.

FA staining. The direct fluorescent-antibody (FA) staining method was used as described previously (21) by utilizing commercially prepared fluorescein-labeled equine antirabies globulin (BBL). The percentage of infected cells was determined after observation of at least 1,000 cells per preparation.

Titration of virus infectivity. The infectivity of rabies virus was determined by one of the following methods: (i) plaque assay technique (15) in agarose-suspended BHK-13S cells, the titers being expressed as plaque-forming units (PFU) per ml; (ii) intracerebral inoculation in adult mice, titers expressed as
mean lethal dose per 0.03 ml; (iii) interference technique in adult mice (used for the HEP strain only). In this last technique, groups of six adult mice were inoculated intracerebrally with tenfold dilutions of HEP virus (0.03 ml) and challenged 14 days later by a second intracerebral inoculation with 50 to 100 mean lethal doses of CVS-26 virus of mouse brain origin. The end point dilution of HEP virus conferring protection against the CVS challenge was calculated and expressed as the infective dose per 0.03 ml.

The titer of VSV stock preparations was determined by plaque assay on BHK-21 cell monolayers under an agarose overlay, and expressed as PFU per milliliter.

Assay and identification of interferon. Tissue culture fluid from infected cell cultures was first dialyzed for 48 hr at 4 C against KCl-HCl buffer, pH 2.0 (12) and then for 24 hr against phosphate-buffered saline, pH 7.3. Interferon content was assayed by treatment of replicate BHK-21 (for hamsters), VH2 (for viper), or GL-1 (for gecko) cell cultures with serial dilutions of test fluids, followed by challenge with 100 PFU of the Indiana strain VSV (18). The titer of interferon is expressed as the reciprocal of the fluid dilution causing 50% reduction in VSV plaque count.

Immunolysis. The susceptibility of cells to immunolysis was determined by the colony inhibition technique (8). Cells dispersed by trypsinization were counted and adjusted to a concentration of 10^4 cells/ml. A 0.4-ml amount of this cell suspension was mixed with 0.2 ml of serum from hamsters immunized with live HEP virus and 0.2 ml of fresh guinea pig serum as complement (1/40 dilution = 2 complement-fixing units) and incubated for 60 min at 36 C. The cells were resuspended in 50 ml of BME and distributed in 5-ml volumes, into ten 60-mm plastic petri dishes. Control cultures were prepared in a similar manner by using heat-inactivated guinea pig serum. Plates were incubated in a 5% CO_2 atmosphere for 8 to 10 days, fixed with methanol, and stained with Giemsa stain. The percentage of lysis was determined by comparing the number of cell colonies in cultures incubated in the presence of active complement with those incubated in the presence of inactivated complement.

Experimental procedure for analysis of chronically infected cell systems. Cell monolayers in 1-liter Blake bottles (ca. 5 X 10^7 cells) were exposed to different strains of rabies virus for 1 hr at a multiplicity of infection of from 1 to 200 PFU per cell (see Results). After 1 hr of adsorption, the virus inoculum was removed and the cultures were washed with PBS, refed with BME, and incubated at 36 C. At the end of 3 to 4 days of incubation, the rabies virus-infected fluid was collected, the cells were dispersed by trypsinization, new 1-liter bottles were seeded with 10^6 cells each, and 60-mm petri dishes, some containing cover slips, were seeded with 5 X 10^6 cells each. This procedure was repeated for several consecutive cell transfers (see Results).

At the time of each subculture, a cover slip was stained with fluorescent antirabies antibody and the percentage of fluorescent cells was determined. Also, the concentration of either released or cell-associated virus, or both, was determined. Monolayers of rabies-infected cells in petri dishes were tested for resistance to superinfection with VSV. After infection with approximately 100 PFU of VSV, the number of plaques formed in rabies-infected monolayers was compared with the number formed in control cultures of the same cell type.

In some experiments, the resistance to VSV of cell cultures chronically infected with rabies virus was measured by the reduction in yield of VSV. At each transfer, rabies-infected and control cell cultures were infected with VSV at a multiplicity of infection of 10 PFU per cell, and the yield of VSV was determined after incubation for 8 hr at 36 C.

In some experiments, the rate of cell multiplication of rabies-infected and control cultures as well as their susceptibility to immunolysis were measured at each transfer.

RESULTS

Detection of infectivity and fluorescent antigen. The results of a series of experiments with Pasteur and HEP strains of virus propagated in Nil-2 cells are summarized in Fig. 1. When the Pasteur strain was used (Fig. 1a), 100% of the cells revealed rabies-specific fluorescent antigen at the time of the first subcultivation. The 100% incidence of cell infection persisted throughout the subsequent six cell transfers. Between the sixth and the seventh passages, however, the incidence of fluorescent antigen-positive cells fell sharply to less than 5%. This low level of cell infection persisted for the next four passages. A new second cycle of infection was indicated by a sharp increase in the incidence of infected cells at passage 12.

The appearance of rabies immunofluorescent antigen changed during the course of the passages; representations of FA-stained cells of the first seven infected passages are illustrated in Fig. 2. Infected cells in early passage exhibited widely dispersed antigen, primarily of a fine particle or granular appearance, and partially localized near the cell membrane, as might be expected from the active viral budding process and susceptibility to immunolysis characteristic of these cell passages (see below). During serial cell passage, rabies antigen was gradually consolidated into larger masses, and the amount of antigen localized at the cell surface diminished (Fig. 2, B-D, passages 2-4). This process culminated in the formation, by the fifth passage, of unusually large inclusions which appeared bizarre and often appeared to undergo spontaneous autolysis within the intact cell (Fig. 2, E, passage 5). Finally, when the period of greatly inhibited cell infection was reached, only a limited number of intermediate-sized antigenic
masses were detected (Fig. 2, F, passage 7). This evolution of the appearance of immunofluorescent rabies antigen was repeated during each consecutive cycle of rising and falling virus production. The titer of released infectious virus reached the highest level after the second passage and then dropped progressively to minimal titers (10 mean lethal doses per 0.03 ml) by the seventh passage. At the time of the fifth and sixth cell transfers, decreases in the yield of released virus were not accompanied by decreases in the incidence of fluorescent antigen-positive cells, but after the seventh cell passage, the further drop in virus yield was accompanied by a marked decrease in the number of cells with rabies antigen.

**Resistance to VSV challenge.** During the first three infected cell passages, cultures were fully susceptible to challenge with VSV. Resistance to challenge with VSV appeared at the fourth cell passage level; the VSV plaque members were reduced more than 90% at a time when all cells contained rabies fluorescent antigen, and the yield of released virus was still relatively high. The resistance to VSV infection declined at the cell passage 11 level at the onset of the second cycle of increasing rabies virus infection. Resistance to VSV infection reappeared after the new cycle of rabies virus infection, appearing after the 11th and 19th cell transfers in a manner corresponding to the pattern established during the first cycle.

Nil-2 cells infected with HEP strain virus and serially subcultivated (Fig. 1b) exhibited behavior similar to that observed in cells infected with Pasteur strain virus (Fig. 1a).

**Detection of interferon.** In cells infected with Pasteur or HEP virus, interferon-like inhibitory activity in low titer was observed in the medium during the first to fourth cell passages, and then reached a titer of 100 or higher at the fifth and sixth cell passages. The inhibitory activity was resistant to exposure to pH 2.0 for 48 hr at 4 C, was not sedimented by centrifugation at 100,000 x g for 2 hr, was resistant to heating at 56 C for 2 hr, and was destroyed by treatment with trypsin (12).

The interferon-like nature of the inhibiting substance was further emphasized by the demonstration that fluids from HEP-infected Nil-2 cell cultures (passage 12) protected control Nil-2 monolayers against superinfection with rabies virus at dilutions up to 1:200 (Fig. 3), whereas the same fluids were ineffective in protecting heterologous species WI-38 human cells against rabies virus.
The possible role of interferon in the restriction of infection in cell passages exhibiting a low level of fluorescence was also indicated by experiments in which replacing the medium four times during a 24-hr period caused the number of infected cells to increase markedly (from 10 to 60%) whereas cultures in which the medium was not replaced exhibited no change. Furthermore, the addition of a known inhibitor of interferon production, actinomycin D (1 μg/ml) to cultures exhibiting a low degree of infection, similarly led to an increase in the percentage of infected cells.

**Resistance to rabies virus challenge.** The resistance of cells to challenge with homologous rabies virus at the time when cultures showed a low level of infection was studied. Monolayers of cells infected with HEP virus at passage 14 (Fig. 1b), showing less than 10% of fluorescing cells, were superinfected with homologous (HEP) virus. Control Nil-2 cells were treated in a similar manner. Twenty-four hours after infection, the superinfected rabies carrier cultures showed no increase in fluorescent antigen-positive cells,
whereas control Nil-2 cultures were 100% infected.

To determine whether this resistance might be caused by selective growth of nonsusceptible cells, the following experiment was performed. Nil-2 cultures chronically infected with HEP virus and showing less than 10% fluorescent antigen-positive cells at the seventh cell transfer level (Fig. 1b) were dispersed by trypsinization, and four cell sublines were established from clones of single-cell origin. Three sublines were free of virus infection and remained virus-free for 12 consecutive cell transfers, whereas one subline was infected with rabies virus and exhibited a characteristic pattern of low and high levels of infection during 12 serial cell passages.

Monolayer cultures from these four cell clones and from control Nil-2 cells were superinfected with PM virus strain. The relative susceptibility of the four cell clones to rabies infection was determined by FA-staining and measurement by plaque assay and titration in mice of infectious virus harvested 72 hr after infection (Table 1). As HEP virus infection is not fatal for adult mice, the newly produced PM virus could be detected selectively by the mouse inoculation technique.

Prior to superinfection, fluorescent antigen was present in 35% of cells from "positive" clone A; all other cultures were free from rabies infection. When cells were examined 72 hr after infection with PM virus, 38% of the cells from clone A showed fluorescent antigen, whereas 100% of the cells were infected in all other cultures, indicating both resistance of clone A to superinfection and the full susceptibility to rabies virus infection of the three virus-free clones. The resistance of the HEP virus-infected cells to superinfection by PM virus was confirmed by the finding that such cells produced sharply reduced levels of mouse-lethal virus.

**Effect of chronic rabies virus infection on cell growth.** The rate of multiplication of HEP rabies-infected and control cells was studied in BHK-21 cultures during 11 consecutive cell transfers. The results indicate that a reduction of cell number from 25 to 60% was usually observed when the percentage of cells containing rabies fluorescent antigen exceeded 10% (Table 2). When the incidence of cell infection was less, the reduction in cell numbers was usually minimal. In the infected BHK-21 cell system, cell-associated virus represented only 1 to 10% of the total virus in the culture. Although both resistance to superinfection with VSV and interferon-like activity in supernatant fluids were demonstrated in the chronically infected BHK-21 cells, no correlation between these phenomena was demonstrable.

**Immunolysis.** The susceptibility of infected cells to lysis by treatment with rabies virus antibody in the presence of complement (S; Wiktor and Koprowski, Bacteriol. Proc.) was studied in chronically infected cell cultures.

BHK-21 cell monolayers were infected with HEP virus at a multiplicity of infection of approximately 200 PFU/cell and tested at regular intervals for susceptibility to immune lysis as well as for production of infectious virus and the presence of fluorescent antigen. The medium was completely exchanged at the time of sampling so that infectious virus titers represented production which occurred after the previous sampling.

Cells were susceptible to immunolysis only during the period of release of infectious virus. The efficiency of cell immunolysis varied directly with the titer of virus released during each period of observation, but was not correlated with the incidence of infected cells determined by FA observation (Fig. 4). The results suggest that susceptibility to immunolysis is dependent upon
TABLE 2. Cyclic expression of viral interference, interferon production, and inhibition of cell multiplication in BHK-21 cells chronically infected with HEP rabies virus

<table>
<thead>
<tr>
<th>Cell transfer</th>
<th>Presence of fluorescent antigen (% of cells)</th>
<th>Infectivity (log 10) PFU/10^6 cells</th>
<th>Reduction of VSV yield (%)</th>
<th>Reduction of cell numbers (%)</th>
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![Graph](http://iai.asm.org/)

**Fig. 4.** Carrier type of rabies virus infection (Flury HEP strain) in hamster cell line BHK-21. Symbols: •, fluorescent-antibody staining; •, virus infectivity; •, susceptibility to immunolysis.

The establishment of strains of cell lines of viper (VSV) and lizard (GL-1) origin chronically infected with rabies virus has been described elsewhere (2). Each cell line was infected with CVS-11 strain rabies virus at an input multiplicity of approximately 1.0, incubated at 33 C, and subcultivated at approximately weekly intervals. An irregular cyclical rise and fall in the titer of released virus with continued cell passage was characteristic of each infected cell system. Released virus titers obtained from VSW cells ranged from approximately 10^4 to 10^6 PFU/ml whereas titers obtained from GL-1 cells varied from <5 to approximately 10^6 PFU/ml.

VSW cells were examined during the 43rd to 52nd consecutive rabies virus-infected passage for the presence of fluorescent rabies antigens, the titer of released virus, resistance of cells to superinfection with VSV, and the presence of interferon in cell supernatant fluids (Fig. 5). The 10 cell passages studied encompassed two cycles of rising released virus production followed by decreasing virus yields. As in the case of hamster cells, the incidence of infected cells determined by FA examination rose in parallel with increased virus release, and the percentage of infected cells remained elevated for one or two passages after the decline in virus release. Rabies virus-infected VSW cells also exhibited cyclically recurring interference with replication of superinfecting VSV, with maximal levels of interference observed in the cell passage in which high levels of rabies cell infection indicated by FA persisted after the decline in infectious virus production. Attempts to demonstrate interferon-like activity in cell supernatant fluids tested at each cell passage were negative (titer = <1:5).

Studies performed during seven consecutive passages of GL-1 cells chronically infected with rabies, from passage 44 to 50, revealed: released virus titers ranging from <5.0 to 2.3 x 10^6 PFU/ml, an incidence of fluorescent antigen-containing cells that was consistently below 5%, and an irregularly varying degree of interference with superinfecting VSV replication ranging from 0 to 99% interference. Again, no interferon-like activity was demonstrated in cell fluids harvested from any passage level (titer = <1.5).
DISCUSSION

A consistent pattern of cyclically rising and falling levels of viral infection was demonstrated in serially subcultivated cells of Syrian hamster cell lines BHK-21 and Nil-2 infected with fixed rabies virus. As indicated by observation of specific immunofluorescent antigen formation, 100% cell infection was achieved within a single cell passage after infection at an input multiplicity of 1 PFU/cell. Maximal yields of released virus were achieved by the second infected cell passage, but a subsequent gradual decline in the level of virus production was not immediately accompanied by a parallel decline in the percentage of infected cells. Infection of the entire cell population persisted through the fourth to the fifth cell passage; during this period, resistance to superinfection with heterologous VSV appeared. Again, resistance to infection with VSV persisted for several cell passages after the decline of both viral production and viral antigen synthesis. Resistance to VSV infection declined concomitantly with the onset of the next cycle of increasing rabies virus infection.

A substance demonstrated in infected cell culture fluids was judged to be interferon-like, on the basis of its (i) resistance to exposure to pH 2 for 48 hr, 56°C for 2 hr, or sedimentation at 100,000 × g for 2 hr; (ii) sensitivity to digestion by trypsin; (iii) reduced production in cells treated with actinomycin D; (iv) efficacy of inhibition against heterologous viruses; and (v) lack of efficacy in cells of heterologous species. Fluctuations in the concentration of interferon in infected cell fluids were observed to correspond with fluctuations in the magnitude of the endogenous rabies infection and in the resistance of cells to superinfecting viruses. Fluctuating rabies virus titers were not the result of the emergence of resistant cell populations. Cell populations clonally derived from uninfected cells harvested during periods of depressed rabies replication remained fully susceptible to rabies virus.

The demonstration of interferon activity in rabies virus-infected hamster cells contradicts previous reports that interferon could not be found in rabbit endothelium cells chronically infected with rabies (6) and human diploid (20) cells in which interference to superinfection with heterologous viruses was exhibited. The reason for this failure to demonstrate interferon in the latter systems is not apparent. However, we recently reported the induction of interferon in human and rabbit cell cultures treated with concentrated rabies virus vaccines (T. J. Wiktor, B. Postic, M. Ho, and H. Koprowski, J. Infect. Dis., in press).

Cyclical changes were also noted in the degree of susceptibility of rabies-infected hamster cells to lysis affected by antiviral antibody in the presence of complement. The degree of susceptibility to immunolysis was found to be correlated with the amount of virus maturation occurring by budding through the cell plasma membrane, at a given stage of cell infection. Virus maturation activity at the cell surface was measured by determinations of the titers of released virus produced, by observing the localization of specific immunofluorescent rabies antigen at the cell surface, and by electron microscopy observations (Y. Iwasaki, T. J. Wiktor, and H. Koprowski, Lab. Invest., in press).

In our previous report of endosymbiotic infection in rabbit endothelium cells, we described an experimental model duplicating the latency observed in natural rabies infections characterized by prolonged incubation periods. We suggest that a synthesis of the findings in this report may lead to a hypothesis explaining how prolonged latent infections may eventually give rise to pathological change and death.

In the infected organism, rabies virus apparently spreads by cell-to-cell contact without cytopathic effect, as observed in infected cell cultures in either the presence or absence of antibody (19). The progression of virus infection may be retarded, but not altogether stopped, by interferon. During this phase of the incubation period, only small amounts of virus will be released into the circulation where it can stimulate immunocompetent cells. Viremia has been demonstrated only occasionally in studies of rabies pathogenesis (11, 16). When circulating antibody titers remain low, as is characteristic of animals observed during the incubation period of rabies, the release of virus may not lead to cell lysis. The concentration of antibody necessary for cell lysis, in vitro experiments, is considerably greater than that required to give demonstrable virus-neutralizing
activity (22). The ultimate induction of rabies disease may then occur when hitherto noncytopathic cell infection progresses to the point where the accumulated, released viral antigen finally stimulates antibody in a concentration high enough to mediate lysis in those cells releasing antigen. The failure of this event to occur until the brain is heavily infected may explain the almost universally fatal outcome of the disease. The possibly critical role of immunolysis in mediating rabies disease is further suggested by the observation that rabies virus-vaccinated experimental animals succumbing to virulent virus challenge frequently die earlier than unvaccinated challenge control animals, and often have higher titers of circulating antibody than do control animals tested at the same time (1, 17).

Finally, we demonstrated in rabies virus-infected viper cells a cyclical rise and fall in released virus titer, in fluorescent antigen concentration, and in the degree of resistance to heterologous virus infection. These patterns are very similar to those observed in rabies virus-infected hamster cells. Unlike the hamster cell system, however, interferon could not be demonstrated in vicer cells. We have not determined whether this failure to detect interferon may be attributed to a different defensive mechanism operative in vicer cells or perhaps only to minor differences in the properties of vicer interferon, causing our technical procedures to be unsuitable for its demonstration. Nevertheless, the prolonged persistence of noncytopathic rabies virus infection in reptile cells is of interest in view of reports of the persistence of infectious rabies virus in the brains of intracerebrally inoculated turtles for periods exceeding 302 days (14).

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LITERATURE CITED