Persistent Infection of Rabies Virus (HEP-Flury Strain) in Human Neuroblastoma Cells Capable of Producing Interferon

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SUMMARY

Apparent interferon-mediated persistent infection of rabies virus (HEP-Flury strain) was established in a human neuroblastoma SYM-I (clone K-104) cell line, which had the ability to produce interferon. This infection produced variable but small amounts of progeny virus and interferon (up to 100 IU/ml), and resisted superinfection with vesicular stomatitis virus (VSV) and Sindbis virus as well as homologous rabies virus. The treatment of this infection with anti-interferon antibody stimulated virus replication and extensive c.p.e. However, some cells survived and grew rapidly without any sign of c.p.e. These produced increased amounts (100 to 1000 times) of infectious and DI particles in the presence of anti-interferon antibody, becoming susceptible to superinfection with VSV but remaining resistant to the original rabies virus. Small plaque mutants appeared and replaced the original virus during the long-term cultivation of the persistent infection. Several mutants tested were all identified as Sdi (DI-resistant) mutants, suggesting that the persisting viruses were endowed by the Sdi mutation with a selective advantage over the original virus even in interferon-mediated persistent infections.

INTRODUCTION

Rabies virus, a member of the rhabdovirus family, has an enveloped, bullet-shaped structure in which a non-segmented, negative-stranded genome RNA is located in a helical form of nucleocapsid with nucleoproteins (Schneider & Diringer, 1976). The virus replicates in the cytoplasm where it induces the formation of specific inclusion bodies which have been identified as clusters of viral nucleocapsids. The mature virions bud from the plasma membrane. The virus produces a weak host cell shut-off and only a mild c.p.e., except in certain virus–host cell systems or culture conditions. The virus tends to establish close interactions with host cells, which resemble endosymbiotic relationships or persistent infections (Wiktor & Clark, 1975).

Many reports have dealt with the persistent infections of rabies virus in some species of cells in culture (Fernandes et al., 1964; Wiktor & Clark, 1972; Kawai et al., 1975; Kawai & Matsumoto, 1977, 1982; Holland et al., 1976; Andzhaparidze et al., 1981; Wild & Bijlenga, 1981), and some authors have identified defective interfering (DI) particles as one of the major regulatory factors involved in establishing and/or maintaining a persistent infection in cultured cells. DI particles have also been shown to play an important role in the evolution of Sdi (DI-resistant) mutants during the long-term maintenance of persistent rabies virus infections (Kawai et al., 1975; Kawai & Matsumoto, 1977, 1982). In these studies the role of the interferon system, another possible regulatory factor in persistent infection systems (Friedman & Ramseur,
obtained from several persistently infected K-104 (RK) cell cultures during long-term cultivation, were cloned by determining the outcome of a rabies virus infection in man and animals, especially during the early phase of infection, has been proposed (Sulkin & Allen, 1975; Stewart, 1981). Although the importance of interferon in a rabies virus infection has been shown by cell culture experiments, its effects on virus-host cell interactions at a cellular level have not been investigated in detail. This is possibly due to a lack of suitable host cells.

Recently, a human neuroblastoma cell line, clone K-104, which originated from the SYM-I strain (Honda et al., 1980) and can produce beta-interferon, was found to be susceptible to rabies virus. Rabies virus induced the production of a significant amount of interferon, which in turn affected the replicative process of the rabies virus in the same culture, ultimately resulting in a significant reduction in the yield of progeny virus (Honda et al., 1984). In our study, we found that the treatment of this cell line with interferon prior to or at the same time as the virus infection influenced this eventual outcome, i.e. the establishment of a persistent infection. Therefore, this cell line appears to be suitable for detailed studies on the rabies virus-host cell interactions in which the endogenous interferon system may participate.

**METHODS**

*Viruses.* The virus stock of the cloned HEP-Flury strain of rabies virus (clone no. 2026/20; Kawai et al., 1975) was prepared by a one-cycle amplification of a mixture of plaque isolates in BHK-21 cell cultures. The amount of DI particle in each sample was determined by the interference focus assay (Kawai & Matsumoto, 1982) before use. The neurovirulent revertant virus derived from rabies HEP-Flury virus was prepared by five serial undiluted passages through suckling mouse brains as described by Clark (1978) and cloned three times by plaque formation. The Sdi mutant of HEP virus (clone no. 2016/4) was obtained from BHK cells persistently infected with rabies HEP-Flury virus (RB-1) after 1 year of cultivation (Kawai & Matsumoto, 1977). Many small plaque mutants obtained from several persistently infected K-104 (RK) cell cultures during long-term cultivation, were cloned by three successive plaque isolations from single plaques. This was followed by one amplification in BHK cells. The stocks of Sindbis virus and VSV (New Jersey serotype) were prepared by passages at low multiplicity of infection (m.o.i.) in BHK cell cultures.

*Cells.* The human neuroblastoma SYM-I cell line, which was originally established by Dr M. Sekiguchi (Institute of Medical Science, University of Tokyo, Tokyo, Japan), was obtained from Dr K. Hayashi (Institute of Medical Science, University of Tokyo), and cloned in our laboratory. Clone K-104 was used throughout this study because of its flat morphology which favours microscopic observation. Uninfected and persistently infected K-104 cell cultures were grown in modified Eagle’s MEM containing twice the normal concentration of amino acids and vitamins and was supplemented with 10% foetal calf serum. Normal and persistently infected (RB cell) BHK cell cultures were maintained in Eagle’s MEM supplemented with 5% bovine serum (BS) and 10% tryptose phosphate broth (Difco). HeLa and mouse L cells were cultivated in Eagle’s MEM containing 5 to 10% BS.

*Infectivity assays.* The infectivity of rabies virus was determined by plaque formation on agarose-suspended BHK cells as described by Sedwick & Wiktor (1967), except that small plaque viruses were counted after a 10-day incubation at 35.5 °C. Plaque assays of Sindbis virus and VSV were performed on monolayers of BHK cells.

*Interference focus assay of DI particles.* The titre of DI particles was determined by the interference focus (IF) formation on BHK cells as previously described (Kawai & Matsumoto, 1982) and measured in IFU/ml.

*Interferons and their assays.* Human interferon (HuIFN) was obtained by inoculating K-104 cells with u.v.-inactivated Newcastle disease virus (NGV; prepared just before the inoculation by irradiating 5 ml of NDV suspension placed on 90-mm glass Petri dish with 50 J/m²/s of u.v. light) and harvesting the serum-free culture medium at the 20th to 30th h of incubation at 36 °C. Mouse interferon (MuIFN) was prepared in the same way by mouse L cells. IFN titres were determined by the plaque reduction or c.p.e. suppression method, using VSV as the challenge virus and HeLa or mouse L cells for HuIFN and MuIFN respectively. The titres were expressed in international units (IU) by calculating the reciprocals of the dilutions that produced a 50% reduction in plaque formation or c.p.e. suppression, and comparing them with the dilutions of standard interferon preparations of HuIFN (NIH, G-023-902-527) and MuIFN (NIH, G-002-904-511) required to give the same effect. The rabies virus contained in the interferon assay samples was removed beforehand by ultracentrifugation at 100,000 g for 60
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 Establishment and properties of persistent infections of rabies virus HEP-Flury strain in human neuroblastoma K-104 cells

In human neuroblastoma K-104 cell cultures, infection with the HEP-Flury strain of rabies virus induced the formation of a significant amount of beta-interferon, which affected virus replication in the same culture, thus resulting in a large reduction in production of progeny virus (Honda et al., 1984). The c.p.e. was not significantly suppressed under these circumstances. The surviving cells eventually began to grow rapidly again to form monolayers, although these recovered cultures were persistently infected. When K-104 cells were treated with interferon before, or at the time of the virus infection, c.p.e. as well as virus replication was suppressed much more, and the infection led directly to a persistent infection.

Once established, the persistent infection in K-104 cells could be maintained by serial subcultures at 3- to 4-day-intervals for at least 200 to 300 cell transfers without eliminating the virus or causing c.p.e. The infected cells multiplied with the same doubling time (about 20 h) as the uninfected control K-104 cells. No significant morphological difference was found between the normal and persistently infected K-104 cells under light microscopy, except that the size and amount of specific inclusion bodies per cell were less than those in BHK cells. The frequency of inclusion-positive cells varied markedly in the early phase of persistent infection, but after 30 to 40 cell transfers, the frequency declined rapidly to approximately 10%, and then did not change markedly during the following 200 to 300 cell transfers (Fig. 1).

Fig. 2 depicts the variation seen in the production of infectivity, DI particles and interferon in the early phase of persistent infection (RK-2). The titres of infectivity and DI particle were 1/1000 or less than those produced by persistent infections in BHK cells. Although the variation of the titre of DI particles was not so regular, the yield of infectivity varied cyclically during these periods. In relation to this observation, the titre of interferon also changed cyclically with almost the same periodicity of that of infectivity. The variation of the frequency of inclusion-positive cells was not so marked in amplitude, but the periodicity was quite similar to that of infectivity during these periods. As mentioned above, K-104 cells were induced by rabies virus replication to produce interferon which in turn suppressed virus replication in the same culture (Honda et al., 1984). We assumed, therefore, that at the initial phase of each periodic cycle of the persistent infection, the amplification of virus genome in K-104 cells was associated with production of interferon and possibly followed by an increase in the level of the antiviral state. Thus, amplification of the virus genome might gradually be suppressed, resulting in a gradual decrease in virus and interferon production. As the variation in DI particle production was not so regular, it is not clear whether DI particles played any role in regulating virus persistence in K-104 cells.

In every case of rabies virus persistent infection in K-104 cells, the production of infectious virus gradually decreased and none could be detected in the culture fluid 1 year after cultivation (after the 100th cell transfer approximately). Infectious virus could be recovered by co-
Y. Honda, A. Kawai and S. Matsumoto

Fig. 1. Changes in the incidence of specific inclusion bodies in the cytoplasm of cells which were infected with rabies virus. The persistently infected RK-2 cells were established by infecting the human neuroblastoma K-104 cells with rabies HEP-Flury virus at an m.o.i. of 4 p.f.u./cell and treating them with 200 IU of human interferon at the time of introduction of the virus infection. The cells were then subcultured serially at 3-5-day intervals for 300 cell transfers. On the first day of each cell transfer, cells grown on a coverslip were fixed and stained to examine the incidence of inclusion-positive cells.

cultivation with BHK cells until approximately the 200th passage. After this time no virus could be recovered by co-cultivation, even though the presence of rabies-specific inclusion bodies was continuously demonstrated by immunofluorescent staining in about 10% of cells. Further, treatment with DEAE-dextran, which was reported to stimulate the production of infectious rabies virus from persistent infection (Wild & Bijlenga, 1981), was not successful in stimulating persistently infected K-104 cells at the late phase (passages 200 to 300) to produce detectable amount of virus.

Interferon as a regulatory factor of the persistent infection in K-104 cell cultures

The periodic production of interferon with almost the same periodicity as production of infectivity (Fig. 2) suggested that interferon might be involved in regulating viral persistence. Other supporting evidence is as follows. Firstly, uninfected K-104 cells were susceptible to both homologous rabies virus and heterologous Sindbis virus, while persistently infected cells (RK-2) resisted superinfection with both viruses (Table 1). On the other hand, uninfected BHK cells were also susceptible to both viruses, but BHK cells persistently infected with rabies virus (RB-3), but which produced no interferon (Kawai et al., 1975), resisted superinfection with only homologous rabies virus, but were highly susceptible to Sindbis virus. Similar results were obtained by the superinfection experiment with VSV (Table 2).

Secondly, as shown in Fig. 3, the persistent infection in K-104 cells (RK-8) was terminated by treatment with anti-interferon antibody. In this experiment, RK-8 cells were used at the early phase of the passage, because the infectivity assay for the RK-2 culture gradually became difficult as the number of cell transfers increased. RK-8 cells at the 18th passage were incubated in the presence or absence of anti-interferon antibody [at a final concentration of 1:200 (15 units/ml)], and subcultured regularly every 3-5 days with a split ratio of 1 in 3, except when c.p.e. was present. In the antibody-treated cultures, the frequency of inclusion-positive cells gradually
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Fig. 2. Comparisons of the titres of interferon, infectivity and DI particles with the incidence of inclusion-positive cells. Culture medium of RK-2 cells (Fig. 1) harvested at each cell transfer from the 30th to 50th cell transfer was assayed for DI virus (a), infectivity (b) and interferon (c) as described in Methods. (d) Incidence of inclusion-positive cells (%).

Fig. 3. Effect of the anti-interferon antibody on the persistent rabies virus infection in K-104 cells. Persistently infected RK-2 cells at the 18th cell transfer were incubated with 15 U/ml of anti-interferon antibody (arrow), and then the cells were subcultured in the presence of the antibody as before. The incidence of inclusion-positive cells and the titres of infective and DI viruses were determined at each time. (a) Incidence of inclusion-positive cells: ○, untreated control culture; ●, anti-interferon antibody-treated culture. (b) ●, infective virus titre in untreated control culture; ○, infective titre in antibody-treated culture; △, DI virus titre in untreated culture; ▲, DI virus titre in antibody-treated culture.

Table 1. Superinfection of persistently infected and normal K-104 and BHK cells

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Yield of progeny virus (p.f.u./ml)</th>
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<tr>
<td>Rabies virus</td>
<td>K-104: $3.3 \times 10^6$; RK-2: $2 \times 10^6$; BHK: $3 \times 10^5$; RB-3: $7.5 \times 10^5$</td>
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<tr>
<td>Sindbis virus</td>
<td>K-104: $3 \times 10^7$; RK-2: $3.3 \times 10^7$; BHK: $7.3 \times 10^5$; RB-3: $2.1 \times 10^8$</td>
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* Confluent monolayers of uninfected K-104 and BHK cells, and persistently infected K-104 cells (RK-2) and BHK cells (RB-3) were grown in 3.5-cm plastic dishes and inoculated with challenge virus at an m.o.i. of 4 p.f.u./cell. After virus adsorption for 60 min at room temperature, dishes were washed with phosphate-buffered saline (PBS) and incubated at 36°C. Culture fluids were harvested after 60 h (rabies virus) or 16 h (Sindbis virus), and assayed for infectivity titre by plaque formation. RK-2 cells were used at the 213th cell transfer, and RB-3 cells were superinfected at the 38th cell transfer.
Table 2. Superinfection of anti-IFN antibody-treated and untreated persistent infections of K-104-cells

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Progeny virus yield (p.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal K-104 cells</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>1.8 × 10^6</td>
</tr>
<tr>
<td>VSV</td>
<td>3.5 × 10^8</td>
</tr>
</tbody>
</table>

* Monolayer cultures of each cell line or subline grown in 3-5-cm dishes were inoculated with rabies virus or VSV at an m.o.i. of 4 p.f.u./cell, and incubated at 36 °C. Culture fluids were harvested at 60 h (rabies virus) or 12 h (VSV) after the virus infection and assayed for their infectivity as described in Methods.

† RK-8 is a K-104 cell culture which was persistently infected with rabies HEP-Flury virus and cultivated in the absence of anti-IFN antibody. The 25th cell transfer was used.

‡ RK-8-R is a subline obtained by cultivating surviving cells of RK-8 culture which had been treated with 15 U/ml anti-IFN antibody at the 18th cell transfer (arrow in Fig. 3). Superinfection was carried out at the 25th day of the antibody treatment (which corresponded to seven additional cell transfers), and superinfected cells were also incubated in the presence of the antibody.

increased and reached the first peak after 7 days (Fig. 3a). As shown in Fig. 3(b) the production of infective and DI particles by antibody-treated RK-8 cultures was also enhanced as compared with the untreated control cultures. This occurred simultaneously with the increase in the number of inclusion-positive cells after the introduction of antibody. C.p.e. appeared on days 10 to 12, and more than two-thirds of the cells died. No morphological change was observed either in the untreated persistent infection during the same period, or in the antibody-treated uninfected K-104 cell cultures. The long incubation periods required to express the effect of anti-interferon antibody were inevitable because at least 5 days were needed for K-104 cells to recover their original susceptibility to Sindbis virus infection following the antiviral state induced by interferon (Y. Honda et al., unpublished data).

The surviving cells of RK-8 in anti-interferon antibody-treated cultures were collected and further cultivated in a medium containing the antibody. They resumed growing after a few days and formed a monolayer. When they were serially subcultured at the same interval as before, the frequency of inclusion-positive cells, once below 20%, began to increase and varied but not so regularly (Fig. 3a). C.p.e. was no longer observed in the subsequent serial cell transfers even though the antibody was present continuously. Much higher titres of infectious and DI particles were produced concomitantly as long as the frequency of inclusion-positive cells was high (Fig. 3b). As shown in Table 2, the recovered culture became almost completely susceptible to VSV, but was still resistant to the homologous rabies virus.

These data indicate that the persistent infection in K-104 cells was regulated by interferon, and that the interferon-mediated antiviral activity was almost completely eliminated by anti-interferon antibodies, and further suggest that the persistent infection achieved after anti-interferon antibody treatment might be regulated by DI particles while in the presence of the antibody (see Discussion).

**Biological properties of rabies virus recovered from persistently infected K-104 cell cultures**

During the course of the long-term maintenance of persistent infection, small plaque viruses began to appear at the 5th to 10th passage of persistent infection, and gradually replaced the original virus. Almost all plaques produced by viruses recovered from the 20th passage were small.

Re-assay of many plaque isolates obtained from small plaques confirmed that small plaque production was genetically determined and stable. Several cloned small plaque mutants isolated from the persistent infection (RK-2) between the 26th and 150th passages were investigated to compare their biological properties with the original large plaque virus and with an Sdi mutant isolated from a DI-mediated persistent infection by the same virus in BHK cells (Kawai &
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Sensitivity to interferon

Preliminary experiments demonstrated that BHK cells could be induced by mouse interferon to enter an antiviral state, although the amount required was ten times or more than that for mouse cells (Fig. 4). Examination in BHK cells of small plaque mutants isolated from the persistently infected K-104 cells demonstrated that their sensitivity to interferon was slightly increased compared with the original wild-type virus (Fig. 4). Repeated experiments using small plaque mutants isolated from several lines of persistent infection also showed similar results, but no interferon-resistant mutant was obtained.

As to the ability of mutants to induce interferon, it was found that the interferon titre was in each case nearly proportional to the yield of infectivity in K-104 cell cultures, i.e. the ratio of the interferon titre to the p.f.u. titre did not differ markedly from that of the original virus (data not shown).

Changes in sensitivity to DI-mediated autointerference

The sensitivity to DI-mediated interference of small plaque mutants obtained from persistent infection (RK-2) was tested by comparison with that of the Sdi mutant obtained from persistently infected BHK cells. The sensitivity was assessed by measuring the ability of each superinfected test virus to replicate in RB-3E, BHK cells that were inoculated in advance with the original large plaque virus and its DI particles and cultivated for several days until the yield of original infectious virus was decreased and when relatively large amounts of DI particles were being produced (as previously described by Kawai et al. (1975)). RB-3E cells were also used as a starting culture from which RB-3, a persistent infection described above (Table 1), was established. Table 3 shows that the superinfesting original virus could not replicate well in these cells because of its high sensitivity to the autointerference caused by DI particles generated by the original virus. However, small plaque mutants isolated from persistently infected K-104 cell cultures as well as Sdi mutant virus (clone no. 2016/4) replicated well in the presence of these DI particles and yielded almost the same amount of infectious virus as obtained from normal BHK cells.
Table 3. Replication of small plaque mutants under the conditions of DI-mediated interference*

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Virus yield (p.f.u./ml)</th>
<th>BHK (A)</th>
<th>RB-3† (B)</th>
<th>% Ratio (B/A)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild HEP-Flury virus (Sdi⁺)</td>
<td>1.5 × 10⁸</td>
<td>9.0 × 10⁶</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>2p-26§</td>
<td>1.9 × 10⁷</td>
<td>1.8 × 10⁷</td>
<td>94.7</td>
<td></td>
</tr>
<tr>
<td>2p-63§</td>
<td>5.8 × 10⁶</td>
<td>4.8 × 10⁶</td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td>2p-150§</td>
<td>7.5 × 10⁵</td>
<td>6.8 × 10⁵</td>
<td>90.7</td>
<td></td>
</tr>
<tr>
<td>2016/4 (Sdi⁻)</td>
<td>1.5 × 10⁷</td>
<td>1.1 × 10⁷</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td>-II</td>
<td>-</td>
<td>1.4 × 10⁷</td>
<td>-</td>
<td></td>
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</table>

* Monolayer cultures of normal and DI particle-containing BHK cells were infected with parent (wild-type) or mutant virus at an m.o.i. of 4 p.f.u./cell. After virus adsorption for 1 h at room temperature, the inoculum was removed, the cells washed three times with warm PBS, and fresh medium was added. Culture fluids were harvested after incubation at 36 °C for 60 h and assayed for their infectivity titre.
† A DI-containing BHK cell culture which was prepared by inoculating rabies wild-type HEP-Flury virus (at an m.o.i. of 3 p.f.u./cell) with its DI particles and cultivating until the fourth cell transfer, when production of infective virus was decreased to the lowest level but that of DI virus was maximum.
‡ Yield of challenged virus from RB-3E culture (B) divided by that from normal BHK cell culture (A) × 100.
§ The same virus mutants as described in Fig. 4.
∥ Not inoculated.

Temperature sensitivity and virulence

The small plaque mutants isolated from persistently infected K-104 cells (RK-2) were only slightly more temperature-sensitive than the original virus, which was initially highly temperature-sensitive and could not replicate well at above 38 °C (Kawai & Matsumoto, 1977). The yield of progeny virus at 33 °C of each small plaque mutant virus listed in Table 3 was 1.5 to 2 times that at 37 °C, while the yields of original virus at 33 and 37 °C were almost the same.

Attempts were then made to examine whether virus virulence changed during the long-term persistence in K-104 cells since virus virulence had been observed with undiluted passages through K-104 cell cultures (Y. Honda et al., unpublished observations). This finding was similar to those reported by Clark (1978) with other strains of neuroblastoma cells. Plaque isolates were prepared from the long-term cultivation of the persistent infection, amplified once in the BHK cell cultures, then examined for pathogenicity by inoculation into the brains of 4-week-old mice. No increase of virulence was found in viruses recovered from the 27th to 99th passages of RK-2 persistent infections of avirulent rabies virus. We also tested whether the virulence of neurovirulent rabies virus decreased after the long-term cultivation of persistent infection in K-104 cells. Persistent infection (RK-3) was established with a neurovirulent revertant virus (see Methods), and several small plaque mutants were isolated during the long-term cultivation of the persistent infection and tested for their virulence. The virulence of mutants did not change during the period of persistence tested (about 1 year), but they all exhibited properties of the Sdi mutant (data not shown).

DISCUSSION

Although there have been many studies on rabies virus persistent infection in cultured cells, only Wiktor & Clark (1972) suggested a regulatory role for interferon in the maintenance of persistent infection in cultured (including BHK) cells. However, they found no association between apparent resistance to VSV superinfection and interferon-like activity in the culture fluids. In fact, BHK cells are believed to be unable to produce interferon, although they can respond to exogenous interferon (Taylor-Papadimitriou & Stoker, 1971; Kawai et al., 1975). Therefore, the role of interferon in rabies virus persistence in BHK cells is still unclear. Our studies with human neuroblastoma K-104 cells furnishes evidence to support the idea that the endogenous interferon may regulate rabies virus persistence in cells capable of its production.

In some other virus-host cell systems, the postulated regulatory role of interferon in virus persistence in cultured cells has been evidenced by experiments using anti-interferon antibody (Inglot et al., 1973; Ramseur & Friedman, 1977, 1978; Youngner et al., 1978). In these studies, however, no attention was given to the fate of the surviving cells in the anti-interferon antibody-
treated cultures, possibly because the treatment was lethal. On the other hand, the treatment of K-104 cells persistently infected with rabies virus by anti-interferon antibody did not result in complete c.p.e., maybe because of the slow-progressing c.p.e. and rapid amplification of DI particles (Fig. 3b). The small number of surviving cells grew rapidly and re-formed monolayers even in the presence of the antibody.

In the recovered culture, however, a new persistent state was established in the presence of anti-interferon antibody; that is, they produced increased amounts of DI particle as well as infectious viruses, and became susceptible to superinfection with VSV while remaining resistant to homologous rabies virus. These properties of the recovered culture resembled those of DI-mediated persistent infection of the same virus in BHK cells (Kawai et al., 1975). As already reported, BHK cells persistently infected with rabies HEP-Flury virus are resistant to superinfection with the homologous virus due to DI-mediated interference. They are highly susceptible not only to the heterologous VSV and Sindbis virus, but also to the Sdi mutant of the rabies HEP virus. Therefore, the resistance to superinfection with the original HEP virus observed in the cells recovered from anti-interferon antibody-treated culture may be due to DI-mediated interference rather than the so-called superinfection exclusion phenomenon (Whitaker-Dawling et al., 1983).

It has been shown, in many virus–host cell systems, that the long-term cultivation of persistently infected cells with many types of viruses generally results in alterations of the virus population, that is, the generation of mutants and their domination over the original virus. This is mainly recognized by changes in the plaque size of the persisting viruses (Youngner & Preble, 1980). In our rabies virus persistent infection in K-104 cells, we also observed the alteration of the virus population, that is, the appearance and replacement of small plaque mutants during the long-term cultivation.

The possible principle of this alteration of virus population in such a closed ecological system as persistent infection in cultured cells may be the evolution of a mutant virus having selective advantage over the original parent virus in a given environment. In our previous studies of rabies virus persistent infection in BHK cells, we found that the DI-resistant (Sdi) mutants appeared and replaced the original virus during the long-term cultivation of the DI-mediated persistent infections owing to their selective advantage. The second virus which replaced the original virus continued to persist with the help of newly generated DI particles produced by the second virus (Kawai & Matsumoto, 1977, 1980). The alteration of the viral population found in the K-104 cells might also be due to the Sdi mutation, because the mutants showed a decreased sensitivity to DI-mediated interference (Table 3). These mutants were also shown to be associated with some other changes in biological properties, such as slightly increased temperature sensitivity and interferon sensitivity (Fig. 4). No interferon-resistant mutant was detected.

Sdi mutations have also recently been demonstrated in other virus–cell systems, such as in persistent infections with lymphocytic choriomeningitis (LCM) virus in MDCK cells, VSV and Sindbis virus in BHK cells and West Nile virus in C3H/RV mouse cells (Jacobson et al., 1979; Horodyski & Holland, 1980; Horodyski et al., 1983; Weiss & Schlesinger, 1981; Brinton & Fernandez, 1983), and recently, in lytic undiluted passage of VSV through BHK cell cultures (Spindler et al., 1982).

Our data show that the selection of Sdi mutants is the main mechanism of the change of the virus population found in long-term persistent infections with rabies virus, even in interferon-mediated viral persistence of K-104 cells. This suggests that such mutations may be involved in the formation of variations in the virus population in prolonged or latent infections in animals. In fact, mutants similar to Sdi have been found in mouse brains in which LCM virus was persistently infective (Jacobson & Pfau, 1980).

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