Growth of Measles Virus in Cultures of Rat Glioma Cells

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The interaction of measles virus with RG-6 cells derived from rat glioma was investigated. When a culture of RG-6 cells was infected with measles virus, the synthesis of viral antigens was detected in very few cells, at most 5%. The apparent resistance to measles virus infection was also repeatedly found in all of the subclonal cells derived from RG-6 cells. Although all of the virus-synthesizing cells had the ability to form plaques on Vero cells, they produced only a reduced amount of infectious virus, i.e., 0.1 plaque-forming units per cell. These results imply the existence of some mechanism that regulates growth of measles virus in cultures of RG-6 cells. The transmission of genetic material of measles virus from infected RG-6 cells to Vero cells was not inhibited in the presence of antiviral serum. This fact may provide a basis for interpretation of the persistence of virus, in the presence of antibody, in patients with subacute sclerosing panencephalitis.

Little is known about the pathogenesis of subacute sclerosing panencephalitis (SSPE), though involvement of measles virus has been shown by several authors (5–7, 15). With regard to the pathogenesis of SSPE, the following two facts should be considered. (i) The isolation of the virus was only successful when trypsin-dispersed brain cells of the patients were cultivated with susceptible cells (1, 8, 9, 12). This seems to show that infectious virus is not produced in the brain cells of the patients. (ii) High titer levels of antibodies against measles virus were present both in sera and cerebrospinal fluids of patients with SSPE (5). It is of interest, therefore, to know how SSPE virus persists in the brain cells of the patients in the presence of antibody.

This report is concerned with the interaction of measles virus with rat glioma cells. We will show that at any time during infection only a part of the cells were infected and viral production in the infected cells was largely restricted. We also describe the effect of antiviral serum on the transmission of measles virus from infected RG-6 cells to uninfected Vero cells.

MATERIALS AND METHODS

Tissue culture. The RG-6 cell line established from rat glioma by Benda et al. (3) was kindly supplied through T. Nakazawa of Keio University School of Medicine, Japan. The cells in monolayer were grown in Eagle minimum essential medium (MEM) with 0.29% L-glutamine supplemented with 10% unheated bovine serum. For virus infection, MEM with 2% heated horse serum (MS) was used.

HeLa-S3 cells were grown in a medium consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract in Earle balanced salt solution, and 10% bovine serum. Through the course of infection, the growth medium was replaced with yeast extract in Earle balanced salt solution with 2% heated horse serum. Vero cells were grown in MEM with 10% calf serum, which was replaced by MS when infected.

Virus. The Edmonston strain of measles virus, which was passaged four to ten times into HeLa-S3 cells, was used. The virus was recovered from the infected cells after three cycles of freezing and thawing followed by centrifugation at 900 × g for 15 min. The clear supernatant fluids were stored at −80°C until used. The infectivity titers ranged from 10^4 to 10^7 mean tissue culture doses (TCID50)/ml.

Infectivity titration. The infectivity was measured either by plaque formation or by tube titration. For plaque formation, a monolayer culture of Vero cells in a petri dish (60 mm in diameter) received 0.2 ml of samples to be tested. After incubation for 2 h at room temperature, the inoculum was removed and the culture was overlayed with 5 ml of agar medium consisting of MEM with 2% heated calf serum. The second overlay was made 4 days after infection, and the plaques were counted on day 8 by staining with neutral red in Hanks solution (1:10,000). For tube titration, the tube cultures of HeLa-S3 cells received a series of 10-fold dilutions of the sample, and the TCID50 was determined by the appearance of cytopathic change on day 14 according to the method of Reed and Muench (14). Three tubes were used per dilution.

Immunofluorescence test. Immunofluorescent cell counting was done according to the method of Kashiwazaki et al. (10). Cover-slip (6 by 30 mm) cultures of RG-6 cells prepared in petri dishes received 0.05 ml of test samples 24 h after the cells were seeded at a concentration of 1.25 × 10^4 cells per dish. After adsorption for 2 h at 37°C in a CO2 incubator, the cover slips were washed and immersed in new petri
dishes containing 5 ml of MS, and incubation was continued. At appropriate intervals, the cover slips were removed from the petri dishes, washed in phosphate-buffered saline, pH 7.2, dried, fixed with carbon tetrachloride, and stained with fluorescent antibody by the indirect method. An adult human serum with staining titer of 1:80 was used as the first serum. Goat anti-human immunoglobulin G labeled with fluorescein isothiocyanate (Hyland Co.) was diluted 80-fold in phosphate-buffered saline and used as the second serum.

Uninfected RG-6 cells were never stained by this procedure, and treatment of measles-infected RG-6 cells with rabbit antiviral serum before addition of the first serum completely inhibited staining of the infected cells, which confirmed that this procedure specially stained measles antigen in infected cells. However, the antigen detected by this staining method was not characterized. The fluorescent cells were counted at a magnification of ×400. Fifty fields were usually counted per cover slip, and the mean number of fluorescent cells per field was obtained. The percentage of cells with fluorescence was determined from counts of 1,000 cells.

Preparation of cell-associated virus. An infected monolayer culture was washed thoroughly and then received the original volume of MS. After three cycles of freezing and thawing, the resulting cell debris was removed by light centrifugation and the clear supernatant fluids were assayed for infectivity.

Antiviral serum. A rabbit was inoculated intravenously with 6 ml of HeLa-S3 cell-grown measles virus at 10^4 TCD_{50}/ml, which was followed by five successive weekly subcutaneous injections of the virus with the same amount of Freund incomplete adjuvant. The animal was bled 1 week after the last injection and the serum was inactivated by heating at 56 C for 30 min. To estimate the neutralization titer of the antiviral serum, serial twofold dilutions of the serum in 0.2 ml were mixed with equal volumes of 100 TCD_{50} of measles virus, and after incubation at room temperature for 30 min, 0.2-ml aliquots of the mixture were added to the tube culture of HeLa cells. The titer, determined after incubation of the culture at 37 C for 14 days, was 1:512. The serum was diluted 40-fold in MS before use.

Cloning of RG-6 cells. RG-6 cells dispersed by 0.1% trypsin plus 0.1% ethylenediaminetetraacetate (EDTA) were plated into the petri dishes at a concentration of 20 cells per dish and cultivated in a CO2 incubator for 2 to 3 weeks. A total of 40 colonies was inserted into test tubes and grown separately.

RESULTS

Immunofluorescent study. When a monolayer culture of RG-6 cells was infected with measles virus at a multiplicity of infection (MOI) of 10 TCD_{50} per cell, visible cytopathic change did not appear even after a prolonged incubation period of 2 weeks. Immunofluorescent staining, however, detected the synthesis of viral antigens in a limited number of cells, 5% at most. Fine granular fluorescent spots appeared in the cytoplasm 24 h after infection; the intensity of the fluorescence was increased thereafter, and the whole cytoplasm became filled with homogeneous fluorescence by 48 h after infection. On the contrary, intranuclear fluorescence could not be detected throughout the course of infection. At any time, the distribution of the fluorescent cells was fairly even and focus-like appearance of the fluorescent cells was rarely found. The time course of the appearance of the fluorescent cells is illustrated in Fig. 1. The fluorescent cells began to appear at 24 h, followed by a rapid increase in the number of fluorescent cells. Maximum levels were reached 30 to 48 h after infection, followed by a gradual decrease. The decrease was due to selective detachment of the fluorescent cells from cover slips caused by degeneration.

Growth of measles virus in RG-6 cells. The tube cultures of RG-6 cells were inoculated with 0.2 ml of virus suspension at an MOI of either 6 or 0.06 TCD_{50} per cell and incubated at 37 C for 2 h. After washing the unadsorbed virus, 1 ml of MS was added to each tube and incubation was continued at 37 C. At the times indicated, both free and cell-associated viruses were tested for infectivity on the monolayer cultures of HeLa-S3 cells.

The virus yields were very poor with either...
MOI and were fairly multiplicity dependent (Fig. 2). Even in the cultures infected at an MOI of 6, the maximum titer of the cell-associated virus was only $10^{2.8}$ TCD$_{50}$/ml and the virus yield per fluorescent cell was roughly calculated to be less than 1.

**Formation of infectious centers by the fluorescent cells.** To further examine the poor yield of infectious virus in RG-6 cells, the following experiment was conducted to see whether all of the fluorescent cells would become infectious centers. The infection of RG-6 cells in tube culture with measles virus at an MOI of 4 TCD$_{50}$/per cell was made as described above. At appropriate intervals, the infected cultures were taken out and divided into two groups. With the first group, the cells were dispersed by 0.1% trypsin and 0.1% EDTA and plated on the monolayer cultures of Vero cells in petri dishes to determine their ability to form plaques. With the remaining group, both the free and the cell-associated viruses were prepared and the infectivities were measured on the monolayer culture of Vero cells by the plaque method. In parallel with these experiments, the replicating tube cultures were taken at 48 h after infection to determine the exact number of infected cells per tube. The cells were dispersed by trypsin and EDTA and washed by light centrifugation. The final cell suspension was smeared on the cover slips, and the number of the infected cells per tube was calculated on the basis of the ratio of immunofluorescent cells to total cells (Fig. 3).

The number of the infectious centers was fairly constant until 48 h after infection. The maximum number of infectious centers was 20,050 per tube, which was recorded at 32 h after infection, and compared with the number of immunofluorescent cells per tube, 21,560, suggesting that all of the fluorescent cells would act as infectious centers. In addition, from these results the amount of virus yield per infected cell was calculated, and the value was again as low as 0.1 plaque-forming units (PFU).

**Susceptibility of subclonal RG-6 cells to measles virus infection.** The preceding results might suggest that there is a genetic difference.

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**Fig. 2. Growth of measles virus in RG-6 cells.** The tube cultures of RG-6 cells were infected with measles virus at a multiplicity of either 6 or 0.06 TCD$_{50}$/per cell. At various times after infection, fluid virus and cell-associated virus were prepared as described in the text, and the infectivities were titrated on the replicating tube cultures of HeLa-S3 cells by the dilution method. Symbols: (○), Cell-associated virus at an MOI of 6; (△), fluid virus at an MOI of 6; (○), cell-associated virus at an MOI of 0.06; (△), fluid virus at an MOI of 0.06.

**Fig. 3. Formation of infectious centers by virus-synthesizing RG-6 cells.** The tube cultures of RG-6 cells were infected with measles virus at an MOI of 4 TCD$_{50}$/per cell, and the infected cultures were divided into two major groups. At the indicated times, the cells in the first group were dispersed by 0.1% trypsin plus 0.1% EDTA. After washing by centrifugation, the resulting cell suspension was plated on the monolayer culture of Vero cells to form plaques by the method described in the text. The number of plaques formed by the cells in the whole tube is plotted (○). With the second group, both fluid virus (△) and cell-associated virus (△) were assayed for infectivity as described in the text.
between fluorescent and nonfluorescent cells. Subclones derived from RG-6 cells were therefore examined for susceptibility to measles virus infection. A total of 40 subclones was grown in tubes, and each was infected with $10^{6.0} \text{TCD}_{50}$ of measles virus. At 48 h after infection, the cells in each tube were dispersed and smeared on cover slips, and the immunofluorescent cell counting was done. Fluorescent cells were recovered from all of the tubes, but the ratio of fluorescent cells to total cells in each culture was again very low, ranging from 1 to 4%. The results clearly show that the culture of RG-6 cells was not a mixture of cells different in susceptibility to measles virus infection.

**Adsorption of measles virus onto RG-6 cells.** An experiment was conducted to see whether the rate of adsorption of measles virus to the culture of RG-6 cells in which the virus growth was largely restricted was different from that found in the culture of HeLa-S3 cells in which virus growth occurred normally. Both cultures of RG-6 cells and HeLa-S3 cells in monolayer were prepared in tubes and inoculated with $10^{4.0} \text{TCD}_{50}$ of the virus in 0.2 ml of MS, and the adsorption was made at 37 C. At the times indicated, the culture fluids containing the unadsorbed virus were removed and the infectivity was measured by the plaque method. The rate of adsorption of measles virus onto the culture of RG-6 cells was quite similar to that observed with HeLa-S3 cells, indicating that the adsorption may proceed normally with every RG-6 cell (Fig. 4).

**Effect of cell density in culture on the efficiency of virus infection.** An analysis was made to explore the poor efficiency of measles virus infection in the culture of RG-6 cells. RG-6 cells were seeded on different days into petri dishes containing the cover slips. The cover-slip cultures at days 1 (sparse culture) and 5 (dense culture) were infected simultaneously with $10^{4.2} \text{TCD}_{50}$ of measles virus. At the times indicated, the cover slips were withdrawn from the sparse and dense cultures, respectively, the immunofluorescent staining was done, and the number of the fluorescent cells per field was counted. The maximum number of fluorescent cells in the sparse culture was three times greater than in the dense culture (Fig. 5). Such a phenomenon, however, was not observed when either HeLa-S3 or Vero cells were infected with measles virus.

**Transmission of virus in the presence of antibody.** Although virus yields of RG-6 cells were shown to be as low as 0.1 PFU per fluorescent cell, all of the fluorescent cells seemed to become infectious centers when plated on Vero cell monolayers. Then it was of interest to know the mode of transmission of the virus progeny from the infected RG-6 cells to the uninfected Vero cells. The tube culture of RG-6 cells was infected with measles virus at an MOI of 4 TCD$_{50}$ per cell. At 7 h after infection, the antiserum was added to the culture and incubation was continued for another 30 min at 37 C. The infected cells were dispersed by trypsin, taken up into the medium containing antiviral serum, plated on the cover-slip cultures of Vero cells, and allowed to settle for 2 h in a CO$_2$ incubator. The cover slips were then transferred into new petri dishes, and further incubation was made in the presence of antibody. On day 3, the cover slips were stained with fluorescent antibody. Syncytia with fluorescence developed under this condition (Fig. 6), and the number of syncytia was fairly proportional to that of the infected RG-6 cells inoculated. The infectivity of the culture fluids, however, could not be detected, whereas an infectivity titer of $10^{4.8} \text{TCD}_{50}$/ml could be recovered from the control experiment in which antibody was omitted. These results revealed that the transmission of the genetic material of the virus from infected RG-6 cells to Vero cells actually occurred even in the presence of antibody that was known to neutralize $10^{6.9} \text{TCD}_{50}$ of measles virus.

![Fig. 4. Adsorption of measles virus onto RG-6 cells. Monolayer cultures of RG-6 cells (○) and HeLa-S3 cells (●) grown in tubes were infected with $10^{4.0} \text{TCD}_{50}$ of measles virus in a 0.2-ml amount and incubated at 37 C. At the times indicated, unadsorbed virus in the fluids was removed and the infectivities were assayed by the plaque method as described in the text.](http://iai.asm.org/)
We have demonstrated that when a culture of RG-6 cells was infected with measles virus, the synthesis of viral antigens occurred in a very limited portion of the cells, 5% at most, and the production of infectious virus per virus-synthesizing cell was as low as 0.1 PFU. Although the majority of the remaining RG-6 cells were protected from the infection by some unknown mechanisms, these uninfected cells were shown not to be genetically different from infected cells in susceptibility to measles virus. In fact, 40 subclones randomly selected from the original RG-6 cells were found to be equally susceptible to infection with measles virus. Furthermore, adsorption of the virus to RG-6 cells proceeded at a rate similar to that obtained with sensitive HeLa cells.

Previous reports about the interaction of measles virus with cell originated from nervous

![Image](http://iai.asm.org/)

**Fig. 5.** Effect of cell density on the appearance of fluorescent cells. RG-6 cells were plated into petri dishes containing cover slips. One-half of the cultures was prepared 5 days ahead of the remaining cultures, which were prepared on the day of infection. The number of cells on the day of use amounted to $1.25 \times 10^6$ and $7.5 \times 10^5$ cells per dish for the sparse (●) and dense (○) cultures, respectively. The cover-slip cultures from both groups were infected simultaneously with $10^{4.3}$ TCD₅₀ of measles virus. Immunofluorescent staining was made at the times indicated, and the number of fluorescent cells was determined as described (11).

**Fig. 6.** Syncytium formation by inoculation of infected RG-6 cells on Vero cells in the presence of antibody. The cultures of RG-6 were infected with measles virus at a multiplicity of 4 TCD₅₀ per cell. At 7 h after infection, antiviral serum was added to the cultures and incubation was made for 30 min. The infected cells were then dispersed by trypsin and suspended in 1 ml of antiviral serum-containing medium. A 0.05-ml amount of the cell suspension was inoculated on the cover-slip culture of Vero cells for 2 h. The cover slips were then placed into new petri dishes and incubation was continued in the presence of antiviral serum. On day 3 after inoculation of the infected cells, immunofluorescent staining was made. Magnification, ×200.
tissues are apparently in conflict with our results in some respects. A culture of hamster dorsal-root ganglion has been shown to support the growth of the Edmonston strain of measles virus for long periods with a limited amount of virus production (13). Similar results have been obtained with human fetal brain cells when infected with either the Shwarz or the Becket ham 31 strain (16). Recently, establishment of long-term infection of African green monkey brain cells was successfully made with the Shwartz strain but not with the Edmonston strain (2). All of these observations may reveal variable interactions between different brain cells and different measles virus strains. One common feature between these observations is, however, that the growth is considerably restricted in the brain cells, suggesting that nervous tissue cells have similar mechanisms for regulating the growth of measles virus. A report demonstrating a nonspecific inhibitor against measles virus growth in brains of rats and human beings appears to support the above postulate (4).

A regulatory mechanism, if exist in the nervous tissue, may not be a single entity. The present experiments may have revealed at least two different mechanisms in the culture of RG-6 cells. The first one may function at an earlier stage of multiplication since the majority of the cells were neither participating in the synthesis of viral antigens nor able to become infectious centers, although measles virus was adsorbed onto them. This type of regulation seems to be influenced by the physiological condition of RG-6 cells because the appearance of antigen-synthesizing cells was more restricted in the dense culture than in the sparse culture. The second one may function at a later stage of infection. This is based on the fact that every antigen-synthesizing cell produced infectious virus only in greatly reduced amounts. This type of regulation at a late step has been reported in infection of hamster embryo fibroblasts with measles virus (11). Two types of infection, lytic and latent, were simultaneously found by these authors (11) when primary hamster embryo fibroblasts were infected with the Schwarz strain. This may also reveal the diverse reactivity of the cells to measles virus. In their established latent culture, 30 to 50% of the cells were found to be synthesizing virus antigen. In our case, an attempt to establish a long-term latent infection was unsuccessful. Subcultures of the infected RG-6 cells resulted in the disappearance of antigen-synthesizing cells shortly after the passage. Thus the regulatory mechanism of RG-6 cells seems to be fairly potent, either single or multiple. A final conclusion on such a mechanism, however, is left for further studies because neither the Schwartz nor the rat-adapted Edmonston strain was examined in RG-6 cells.

The transmission of the genetic material of measles virus from infected RG-6 cells to uninfected Vero cells was not prevented by antiviral serum. Since no virus infectivity could be recovered from the fluid of the culture in which antiviral serum was contained, it seemed certain that the transmission of viral genome in the presence of antibody was not mediated through the virus released from infected RG-6 cells. Further, the potency of the antiviral serum was high enough to inactivate as much as 10^4-6 TCID_{50} of measles virus at the concentration used, whereas the infectivity associated with infected RG-6 cells was calculated to be as low as 68 PFU per cover-slip culture; these facts strongly suggest that involvement of the cell-associated virus in the transmission was also unlikely.

As the mode of transmission of genetic material in which neither released nor cell-associated virus is involved, three mechanisms might be considered: transmission via intercellular processes, direct transmission by cell fusion, and ingestion of infected RG-6 cells by Vero cells. By which of them the virus transmission from RG-6 cells to Vero cells was mediated remains to be determined. In any case, however, the failure of antibody to block the transmission of measles virus genome from cell to cell may reflect the fact that measles virus persists for a long time in the brains of SSPE patients in spite of the presence of high antibody titer in serum and cerebrospinal fluid.

**LITERATURE CITED**


