Isolation and Persistence of Chikungunya Virus in Cultures of Mouse Brain Cells

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SUMMARY

Cultures of brain cells taken from mice at 1 to 3 days after peripheral infection with Chikungunya virus, showed loss of contact inhibition and morphological alterations which suggested that these cells may have been transformed. In cultures infected in vitro with a low dose of about $10^6$ LD$_{50}$/ml of culture medium, areas of epithelial-like cells and hypertrophied cells were dominant, cultures were destroyed after infection with a high dose of about $10^6$ LD$_{50}$/ml of culture medium. Cultures not destroyed by virus were studied for up to 4 months. The supernatant fluids of these cultures were titrated regularly in an adult mouse system in which the amounts of virus recorded varied from $10^3$ to $10^6$ LD$_{50}$/ml.

INTRODUCTION

Chikungunya (CHIK) virus, a member of the alphavirus group of togaviruses, produces a dengue-like illness in human beings (Ross, 1956; Ranitz et al. 1965). It does not cause an acute encephalitis but, as it has a viraemic period, it almost certainly infects CNS tissue. In view of the recent discoveries that viruses and virus-like agents play a part in chronic CNS disease such as progressive multifocal leucoencephalopathy (PML) (Padgett et al. 1971; Weiner et al. 1972), subacute sclerosing panencephalitis (SSPE) (Baublis & Payne, 1968; Horta-Barbosa et al. 1969), Creutzfeldt-Jakob disease, and kuru (Gibbs & Gajdusek, 1970), we feel that CHIK virus has a potential as a cause of chronic CNS disease of humans in the field and also of animals in the laboratory.

Tick borne togaviruses have already been incriminated in chronic CNS disease causing psychological and neurological abnormalities (Freymann, 1957). The possibility that others might be involved should not be overlooked, particularly as it is well established that togaviruses can survive in various animal organs, including nervous tissue, for long periods of time (Reeves, 1961; Webb, 1969). Chronic neurological diseases, such as early dementia, occur in the indigenous populations of the areas in which tick borne and alpha togaviruses are active, although the aetiological mechanisms involved have not been investigated extensively. Since it has been established already that viruses or virus-like agents are involved in such diseases, and even in the process of ageing (Gajdusek, 1972), we are studying the potential of viruses to survive in the CNS and to effect the glial elements. We show that CHIK virus may be particularly interesting for this work.
METHODS

Virus. Chikungunya virus was obtained in its 174th mouse brain passage as a 10% mouse brain suspension in sterile bovine albumin phosphate saline (BAPS) at pH 7.0 from Dr C. J. Bradish of the Microbiological Research Establishment, Porton, Salisbury.

Mice. Statistically random-bred, barrier maintained specific pathogen free mice of either sex of the Swiss A2G strain were used in all the experiments. Six-day-old mice were used for brain cell cultures and 3- and 4-week-old mice for titration of virus.

Cell culture. Three litters, each containing ten baby mice, were inoculated intraperitoneally (i.p.) with 0.05 ml of CHIK virus containing approx. 10^6 LD_{50}/ml. At the time of inoculation the litters were respectively 3, 4, and 5 days old so that on post-inoculation days 1, 2 and 3 a litter of 6-day-old mice was available for cell culture. Brain cells from one litter were pooled and cultured on each post-inoculation day up to the fourth when all the mice had died.

Brain cells were also cultured from normal uninfected 6-day-old mice. Some of these cultures were used as uninfected controls and some infected when confluent, after 8 days growth, with either 10^6 LD_{50}/ml of culture fluid or 10^4 LD_{50}/ml of culture fluid. Trypsin dispersed suspensions of whole brains were prepared by the method of Youngner (1954) as adapted by Illavia & Webb (1969). Plastic Falcon bottles (30 ml) were seeded with 5 ml of the cell suspension and Sykes-Moore chambers (Bellco glass Incorp., New Jersey, U.S.A.) were filled with the suspension which contained 5 × 10^5 cells/ml. The cell cultures were incubated at 36 °C and examined daily. Over the first few post-culture days the fluids from the Falcon bottles were titrated independently for virus infectivity. As similar infectivities were recorded, only pooled fluids from the Falcon bottles were titrated on the later days. Assays of infectivity were made every 3 to 7 days when the culture fluid was replaced with fresh medium after washing the cultures with phosphate-buffered saline.

Assay of virus infectivity. Serial tenfold dilutions of cell culture supernatant medium were inoculated into groups of five 3- to 4-week-old Swiss A2G mice; four serial dilutions being used for each titration. Each mouse received 0.03 ml by the intracerebral (i.c.) route. The LD_{50} was calculated by the method of Reed & Muench (1938). Samples from the brains of inoculated mice were kept so that the virus could be checked by the complement fixation test for its similarity to that originally inoculated into the cultures of mouse brain cells.

RESULTS

When brains were taken 1 and 3 days after infection, the cells had formed a confluent sheet after 6 days in culture which remained until the experiment terminated at 118 and 89 days, respectively. Brain cells taken 2 days after infection took 23 days to form a confluent sheet and showed some shedding of cells and regrowth after 46 and 54 days in culture. This variation was not explained.

Virus was present in the cell culture fluids throughout the experiment. Table 1 shows the range of virus infectivities detected. Culture fluid initially containing 10^6 LD_{50} of virus/ml was found to contain no infective virus after incubation for 24 h at 36 °C, indicating that virus was replicating continuously in the cell culture system.

In contrast to the uninfected cultures, numerous areas of epithelial-like cells were identified in infected cultures within 2 days. In some areas cells overlapped and piled up (Fig. 1a to c), indicating loss of contact inhibition. These areas appeared within 3 days in culture and remained throughout the experiment as clear evidence of an excessive proliferation
**Table 1. Virus infectivities in supernatant fluids from mouse brain cells infected in vivo and in vitro**

<table>
<thead>
<tr>
<th>Supernatant fluid of in vivo infected cell cultures</th>
<th>Days of infection</th>
<th>Infectivity of virus at seeding in log (LD_{50}/ml of cell suspension)</th>
<th>Range of virus infectivities in log (LD_{50}/ml)</th>
<th>Time over which virus was assayed (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st after inoculation</td>
<td>1.9</td>
<td>4.0–6.1</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>2nd after inoculation</td>
<td>3.5</td>
<td>3.7–6.0</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>3rd after inoculation</td>
<td>4.3</td>
<td>3.8–6.0</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>8th day of growth</td>
<td>6.2</td>
<td>1.7–6.0</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>8th day of growth</td>
<td>4.2</td>
<td>3.7–6.0</td>
<td></td>
<td>31</td>
</tr>
</tbody>
</table>

![Image](image_url)

Fig. 1. Brains taken from mice 3 days after infection with CHIK virus, after eight days of culture. *(a)* appearance of epithelial-like cells showing increased cell density. Areas of piled-up cells where contact inhibition has been lost *(B)*. Fibroblast-like cells *(C)*. Magnification × 32. *(b)* typical areas of piled-up cells where contact inhibition has been lost. *(c)* epithelial-like cells showing increased cell density surrounded by fibroblast-like cells. Magnification × 125; stain, Giemsa.
which was absent in uninfected cultures. Fibroblast-like cells were present in the early stages but the majority of these disappeared within the first 15 to 20 days. During this time the areas of epithelial-like cells increased in size and formed a much denser cell growth than that in uninfected cell cultures (Fig. 2a, b). It was of interest to note that the pH of the culture medium from infected cultures changed from 7.4 to 6.5 when tested over a week, whereas over the same period the pH in uninfected cultures fell to only 7.2. These pH values appeared not to alter the virus infectivity significantly.

The amount of CHIK virus detected in the cell culture supernatant fluids from the Falcon bottles infected in vitro when confluent with $10^{6.2}$ and $10^{4.2} \text{LD}_{50}$/ml of CHIK virus, is shown in Table 1. On the day of infection the cells were predominantly fibroblast-like. At one day after infection by $10^{6.2} \text{LD}_{50}$ the cells showed signs of destruction with many detached cells and about 75% of the sheet intact. Over the next four days the cell sheets were further destroyed to leave only a few cells attached at 5 days after infection. A few
cells remained attached until the 21st day. Over the first five days, \(10^{6.7}\) to \(10^{8.0}\) LD₅₀/ml of virus was detected in the culture fluids. It then fell sharply to \(10^{5.7}\) LD₅₀/ml seven days after infection with virus. Eight days later virus was still present but no virus was detected 17 or 21 days after infection of the cultures.

The monolayers infected with \(10^{4.2}\) LD₅₀/ml of culture fluid showed slight shedding of cells in the early stages of the experiment. At 5 days after infection the appearance of the cell sheet was very different from the non-infected control cultures (Fig. 3a). Large round cells were scattered amongst the less well defined fibroblast-like cells (Fig. 3b, 4). Small

Fig. 3. (a) uninfected mouse brains showing fibroblast-like cells. (b) and (c) mouse brain cells infected 8 days after culture with \(10^{4.2}\) LD₅₀ of virus/ml of culture fluid. (b) 5 days after infection. Note large round cells (A) scattered amongst the less well-defined fibroblast-like cells (B). (c) 7 days after infection. Note large round cell (A) and area of epithelial-like cells (B). Magnification × 125; stain, Giemsa.
Fig. 4. Mouse brain cells infected after 8 days' growth with $10^{4.2} \text{LD}_{50}$ of CHIK virus/ml of culture fluid. Cells at 15 days after infection. Note (a) hypertrophied cells (A). Area of epithelial-like cells (B). Magnification $\times 32$. (b) Epithelial-like cells. (c) Hypertrophied cells. Magnification $\times 125$; stain, Leishman.

areas of greater cell density which were present at the 5th day were clearly epithelial-like by the 7th day (Fig. 3c). By the 15th day hypertrophied cells and multiple areas of epithelial-like cells dominated the cell sheets (Fig. 4a to c) and remained so until the experiment was terminated at 42 days because of bacterial contamination.

Virus liberation after infection at the lower level of $10^{4.2} \text{LD}_{50}$/ml ranged from $10^{4.7}$ to $10^{6.0} \text{LD}_{50}$/ml of culture fluid. It is interesting to note that the amount of virus detected in the culture fluids on the first day of infection closely resembled the original inputs of virus.

The uninfected control cultures became confluent by the 8th to 10th day and remained
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so throughout the experiment. The cells were predominantly fibroblast-like (Fig. 3a) and supernatant fluids from control cultures showed no detectable virus infectivity.

The above experiments were repeated on nine separate occasions with similar results.

**DISCUSSION**

The interest in this paper lies in the long survival of CHIK virus infectivity in cultured mouse brain cells, and also in the unusual proliferative changes. Studies of cultures of mouse or sheep brain cells have shown that infection with scrapie increased growth potential (Field & Windsor, 1965; Gustafson & Kanitz, 1965; Haig & Pattison, 1967), and Caspary & Bell (1971) suggested that individual cells were more active than normal cells, and resembled tumour cells. When cells from the brains of animals infected with the agents of kuru, Creutzfeldt–Jakob disease, or mink encephalopathy are cultured, the glial cells also seem to be stimulated (Marsh & Hanson, 1969; Haig & Clarke, 1971; Gajdusek et al. 1972).

Zlotnik (1968) has also shown that a number of peripherally inoculated togaviruses cause both hypertrophy and proliferation of astrocytes as one of the earliest histopathological changes. Electron microscope studies carried out by Boulton et al. (1971) have shown that the astrocytes and oligodendrocytes predominated in mouse brain cell cultures infected with Langat virus and were the only cells seen infected with virus.

Transformed areas have recently been seen in subcultures cells derived from the brain of two patients with SSPE (Katz, Koprowski & Moorhead, 1969) and in the brain from a patient with Creutzfeldt–Jakob disease (Hooks et al. 1972). In the latter, particles morphologically resembling the oncogenic RNA viruses were detected by electron microscopy. More recently, ter Meulen et al. (1972) observed morphological changes and abnormal growth in fused-cell cultures containing multiple sclerosis brain tissue carrying a virus of parainfluenza type. Webb, Illavia & Laurence (1971) studied a human foetal brain culture deliberately infected with attenuated strains of measles virus and observed loss of contact inhibition and colony formation. Similar results were obtained using the viruses of West Nile, Kyasanur Forest disease and Langat (Illavia & Webb, 1972).

The present results show that CHIK virus also has a marked proliferative effect on non-neuronal brain cells combined with continuous virus production. This was especially true in cell cultures taken from mice one to 3 days after peripheral infection with CHIK virus, when loss of contact inhibition, morphological alteration and a lowered pH of the medium, suggested that these cells may have been transformed by CHIK virus. In contrast, cultures infected with the larger dose of virus were rapidly destroyed whereas those infected with the lower dose of virus continued growing and appeared similar to cultures prepared from infected brains. Their appearance was similar to that seen with SV 40, an oncogenic DNA virus, when inoculated into cultures of primary mouse kidney cells (Black, 1968). In this context it is of interest that a papovavirus similar to SV 40 has been isolated from patients with PML (Weiner et al. 1972). However we do not think that SV 40, or a similar agent, is a contaminant contributing to the changes we report since these have not been detected by extensive electron microscope studies carried out in this laboratory on brain tissue and cells infected with CHIK, Sindbis or Langat virus (Boulton et al. 1971; S. Pathak, personal communication, 1973). The reported changes in the infected mouse brains did not occur in uninfected control cells or in mouse brains infected with other viruses, such as virulent or avirulent strains of Semliki Forest virus (S. W. Precious and H. E. Webb, unpublished observations).
Thus CHIK virus may be particularly valuable in further studies on the glial response in chronic CNS diseases.

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REFERENCES


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