Cell Fusion by HeLa Cells Persistently Infected with Haemadsorption Type 2 Virus

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SUMMARY

When a monolayer culture of HeLa cells persistently infected with haemadsorption type 2 virus (HeLa/HA2) was dispersed by trypsin and plated on a monolayer culture of uninfected HeLa cells, syncytia developed. For induction of the syncytia, it was specifically pre-requisite to treat the carrier cells with trypsin. The formation of syncytia started at 1 h and was completed by 4 h, followed by the synthesis of virus antigens in the syncytia. The presence of cycloheximide at a concentration of 50 μg/ml did not inhibit the syncytium formation but the presence of antiviral serum completely suppressed it. The results indicated that the principle responsible for the cell fusion is not only of virus nature but also localized in a masked form on the surface of the carrier cells and that the trypsin treatment efficiently activates it. The mechanism of cell fusion in this system and the applicability of the above procedure to the isolation of causative agents from the lesions with which paramyxovirus-like structures are latently associated are discussed.

INTRODUCTION

Cytopathic effects of virulent viruses are thought to occur in two ways. The first could be due to lysis of individual cells and the second one is due to syncytium formation as observed in cells infected by herpes simplex (Roizman, 1962), mumps (Henle, Deinhardt & Girardi, 1954), measles (Enders & Peebles, 1954), Newcastle disease (Johnson & Scott, 1964) and parainfluenza (Lépine et al. 1959; Compans et al. 1964) viruses. Although the latter is known to occur by fusion of the virus infected cells to uninfected cells, the mechanism is not clear.

Infection of HeLa cells with haemadsorption type 2 virus (HA2) readily establishes a virus carrier state designated as HeLa/HA2 without gross appearance of cytopathogenicity and the carrier cells can be passaged serially by the aid of trypsinization (Ishida et al. 1964). As HA2 virus is capable of inducing late cell fusion (Okawa et al. 1970), some protecting mechanism against cell fusion may be operating in the carrier culture. Recently, one of the authors found that L cells infected with egg-grown Sendai virus closely related to HA2 virus were not infectious for L cells by themselves, but became infectious after trypsinization (Homma, 1971). It was also found that such trypsinized infected cells acquired simultaneously the ability to fuse monolayer cultures of Vero, HeLa and FL cells when plated on the latter cells.

In view of these facts, the present experiments were attempted to unmask the fusing

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ability of HeLa/HA2 by the aid of trypsin treatment and it was hoped that this procedure would be applicable to isolation of viruses from the tissues where paramyxovirus-like structures have been found (Chou, 1967; Györkey et al. 1969; Meulen et al. 1972; Prineas, 1972).

**METHODS**

**Cell cultures.** Procedures used to grow and maintain HeLa/HA2 culture were as described previously (Ishida et al. 1964). For the growth of HeLa/HA2 and uninfected HeLa cells, a medium consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 0.45% glucose in Earle's balanced salt solution (YLE) with 10% unheated bovine serum was used. As a maintenance medium (MS) for both cultures, YLE supplemented with 2% heat inactivated horse serum was employed.

**Virus and virus assay.** For infection of HeLa cells, the 59-34 strain of HA2 virus was used. The virus was prepared by infecting primary cultures of monkey kidney cells and collecting the culture fluids after low speed sedimentation. The virus was titrated on tube cultures of primary monkey kidney cells (Miyamoto, Homma & Ishida, 1967) and TCD₉₀ was calculated according to the method of Reed & Muench.

**Antiviral serum.** A hyperimmune serum against Sendai virus (which is known to have antigens common to HA2 virus) (Chanock et al. 1958), was prepared in rabbits. A 1 ml sample of the allantoic harvest of Sendai virus containing 10⁴ H.A.U. per ml was injected intravenously twice a week into a rabbit. A total of 10 successive injections was made and the animal was bled 7 days after the last injection. The serum was inactivated at 56 °C for 30 min and stored at -20 °C until used.

**Trypsinization of the carrier cells.** A monolayer culture of HeLa/HA2 cells was washed once with phosphate-buffered saline (PBS), pH 7.2 and then treated with a mixture of 0.05% trypsin (Nutritional Biochemicals Corp., 1:300) plus 0.05% EDTA in PBS. After aspiration of the mixture, the cells were incubated for 3 min at 37 °C and the detached cells were dispersed by gentle pipetting in MS.

**Fusion assay.** A number of carrier cells suspended in MS was plated on tube cultures of complete monolayer of HeLa cells and incubated at 37 °C. At the times indicated, the cells were dispersed and cell counts were made. Fusion index was expressed by the following formula. Fusion index: (cell number in control sample/cell number in test sample) - 1 (Okada & Tadokoro, 1963).

**Quantitative haemadsorption test.** This test was applied to the experiment described in Fig. 6 where amounts of red blood cells adsorbed on the infected cells were measured by a modification of the method described elsewhere (Nagai, 1973). Test samples prepared in tubes were washed twice with 5 ml of PBS and 1 ml of 2% guinea pig erythrocytes added. They were left at 4 °C for 20 min and unadsorbed erythrocytes were removed by washing thrice with 5 ml of PBS. Adsorbed erythrocytes were ruptured by the addition of 1 ml of distilled water and centrifuged 900 g for 5 min. The extinction of the haemoglobin released into the supernatant fluids was read at 575 nm.

**RESULTS**

**Syncytium formation by the carrier cells**

When the carrier cells were dispersed by a mixture of 0.05% trypsin and 0.05% EDTA and then plated on an uninfected monolayer culture of HeLa cells, syncytia developed within a few hours after incubation at 37 °C (see Fig. 4). The size of the syncytium depended
Cell fusion by HA2 virus carrier cells

Fig. 1. Syncytium formation induced by the carrier cells. A monolayer culture of HeLa/HA2 was dispersed by 0.05% trypsin plus 0.05% EDTA and the suspension containing $2 \times 10^9$ cells per 0.05 ml of MS was inoculated on a cover slip (6 × 30 mm) culture of HeLa cells. Giemsa staining was made at 5 h after inoculation of the carrier cells. Magnification × 100.

Fig. 2. A linear relationship between the number of the carrier cells inoculated and that of syncytia. A series of twofold dilution of the carrier cells dispersed by 0.05% trypsin plus 0.05% EDTA was inoculated on the cover slip cultures of uninfected HeLa cells (O–O) or the carrier cells ( – – – – ). Giemsa staining was made at 5 h after inoculation of the carrier cells and the number of syncytia per cover slip was determined by microscopic examination.

on the number of the carrier cells inoculated. Roughly, when the number of the carrier cells inoculated was more than 50% of the recipient cells, the whole culture became a single giant syncytium and when fewer carrier cells, i.e. 1 cell per 200 recipient cells, were applied, syncytia quite homogeneous in size containing 30 to 40 nuclei per cell were formed (Fig. 1). In the latter case, the number of syncytia was roughly proportional to the number of the inoculated cells (Fig. 2). The syncytium never appeared when the carrier cells were inoculated on monolayer cultures of the carrier cells instead of uninfected HeLa cells, indicating that the formation of the syncytium occurred only between the carrier cells and uninfected HeLa cells.
Fig. 3. Requirement of trypsin treatment of the carrier cells for syncytium formation. A monolayer culture of the carrier cells was dispersed by 0.05% trypsin (○○), 0.05% EDTA (▲▲) or vigorous pipetting (■■) and the indicated cell number was plated on replicate cultures of HeLa cells. Fusion indices were determined after incubation for 5 h.

Table 1. Effect of antiviral serum on the syncytium formation

<table>
<thead>
<tr>
<th>Dilution of antiviral serum</th>
<th>Fusion index</th>
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<tr>
<td>1:10</td>
<td>0.12</td>
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<tr>
<td>1:40</td>
<td>0.15</td>
</tr>
<tr>
<td>1:160</td>
<td>0.93</td>
</tr>
<tr>
<td>1:640</td>
<td>1.01</td>
</tr>
<tr>
<td>No antiviral serum</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Requirement of pre-conditioning of the carrier cells for syncytium formation

The following experiment demonstrated that trypsin treatment of the carrier cells was required to induce syncytium formation. The carrier cells in monolayer were dispersed either by vigorous pipetting or EDTA or trypsin alone and the cells at the indicated number were plated on replicate cultures of HeLa cells. As shown in Fig. 3, syncytium formation was not evident in cultures which received the carrier cells dispersed by pipetting or EDTA, while a considerable degree of syncytium formation could be observed when the carrier cells were treated with trypsin.

Effect of antiviral serum on syncytium formation

As described before, the carrier cells have virus antigens at any given time on the cell surface and these can be readily detected by haemadsorption and immunofluorescent staining (Ishida et al. 1964). On the basis of this fact, it was thought that the virus antigens localized on the surface of the carrier cells might be responsible for syncytium formation. Samples of 10^5 trypsin-dispersed carrier cells were added to a series of fourfold dilutions of virus antiserum and the whole was plated on replicate cultures of HeLa cells. As shown in Table 1, lower dilutions of the antiserum strongly inhibited syncytium formation.
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Fig. 4. Time course of syncytium formation by the carrier cells. Samples of $10^5$ carrier cells dispersed by trypsin were plated on replicate cultures of HeLa cells and fusion indices were recorded at hourly intervals.

Fig. 5. Time course of the appearance of haemadsorption in the syncytium induced by the carrier cells. The suspension containing 300 carrier cells after dispersion by trypsin was inoculated on replicate cultures of HeLa cells in 60 mm Petri dishes. Haemadsorption was made at the indicated times and the number of syncytia which could be detected as discrete haemadsorption foci was counted macroscopically (Homma, 1971).

**Time course of syncytium formation**

Samples of $10^5$ carrier cells dispersed by trypsin plus EDTA were plated on replicate cultures of HeLa cells and the time course of syncytium formation was followed. As illustrated in Fig. 4, syncytium formation began to occur within 1 h after plating and maximum levels were reached at about 4 h. On the other hand, the results from a parallel experiment, where the time course of haemadsorption in the syncytia was followed, showed that the syncytia became haemadsorptive only after 1 day (Fig. 5).

**Effect of cycloheximide on syncytium formation**

An experiment was conducted to see whether the synthesis of protein is required for the induction of syncytium formation by the carrier cells. Various concentrations of cycloheximide, a potent inhibitor for protein synthesis, were added to the medium containing $10^5$ trypsin-dispersed carrier cells and the mixtures were inoculated into the cultures of HeLa cells. After incubation for 5 h at 37 °C, the fusion indices were recorded. Table 2 shows that cycloheximide even at a concentration as high as 50 μg per ml did not inhibit syncytium formation. A parallel experiment showed that cycloheximide at a concentration of 2 μg per ml was enough to induce a complete inhibition of the synthesis of new progeny virus in HeLa cells primarily infected with monkey kidney grown HA2 virus. The result together with those obtained from the foregoing experiments revealed not only that syncytium formation by the carrier cells did not require virus replication but also suggested that preformed virus antigens on the carrier cells were exclusively responsible for the phenomenon under study.
Table 2. *Effect of cycloheximide on the syncytium formation*

<table>
<thead>
<tr>
<th>Cycloheximide (µg/ml)</th>
<th>Fusion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.27</td>
</tr>
<tr>
<td>0.4</td>
<td>1.16</td>
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<td>10.0</td>
<td>1.05</td>
</tr>
<tr>
<td>50.0</td>
<td>1.16</td>
</tr>
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</table>

Fig. 6. Time course of haemadsorption and syncytium forming ability of HeLa cells primarily infected with HA2 virus. Monolayer cultures of HeLa cells were infected with monkey kidney grown HA2 virus at a multiplicity of 25 TCD50 per cell. After washing the unadsorbed virus, half of the cultures were fed with YLE and half with MEM. With a portion of the latter cultures, MEM was replaced by YLE at 24 h after infection. In this particular type of experiment, the 'quantitative haemadsorption' was made as described in Methods, at the times indicated, and the ability of cell fusion was measured by plating the infected cells after dispersion by trypsin on monolayer cultures of HeLa cells. The cell number was adjusted to 2 x 10⁶ cells per tube and the fusion indices were estimated after incubation for 3 h. •—•, fusion index in YLE; ○----○, haemadsorption in YLE; ▲——▲, fusion index in MEM replaced by YLE at 24 h; △——△, haemadsorption in MEM replaced by YLE at 24 h; ■——■, fusion index in MEM; □——□, haemadsorption in MEM.

**Syncytium formation by HeLa cells primarily infected with HA2 virus**

HeLa cells used in this experiment were not able to support the growth of Newcastle disease virus in a serine-free medium such as Eagle's minimum essential medium (MEM). In MEM, the synthesis of virus RNA and virus specific-antigens proceeded normally but neither haemagglutinin nor haemadsorption was demonstrable until external serine was supplied (Itoh et al. 1969). These observations were repeated with HA2 virus. Monolayer cultures of HeLa cells were primarily infected with monkey kidney grown HA2 virus at a multiplicity of infection of 25 TCD50/cell and incubated for 2 h at 37 °C. After washing away the unadsorbed virus, half of the cultures were fed with YLE and half with MEM, both containing 2% dialysed horse serum. With a portion of the latter cultures, MEM was replaced by YLE at 24 h. At various intervals, cultures from the respective experiments were taken up and tested both for haemadsorption and cell fusion activity. As illustrated
in Fig. 6, the cells cultured in YLE became haemadsorptive at 24 h and simultaneously acquired the ability to mediate cell fusion, while those cultured in MEM remained non-haemadsorptive and never developed the ability to mediate cell fusion through the entire period of the experiment. When MEM was replaced by YLE at 24 h, the cells immediately acquired both activities. The results suggest that the appearance of the virus antigens on the cell surface is responsible for the cell fusion induced by the infected cells.

DISCUSSION

The present experiments showed that syncytium formation by the carrier cells was mediated through preformed virus antigen(s) localized on the cell surface. The antigen was in a masked form and trypsin treatment of the carrier cells was specifically required to activate it. Even after activation by trypsin, syncytium formation occurred only between the carrier cells and uninfected cells, showing that some protecting mechanism against cell fusion may exist in the carrier cells. Hence, the masking of the antigen may not be essential for maintenance of this carrier state but it might be functioning in the process of establishing the carrier system by protecting uninfected cell populations against cell fusion.

In HeLa cells primarily infected with HAz virus, although cell-fusing ability appeared simultaneously with haemadsorbing ability (Fig. 6), the latter was not influenced by trypsin treatment, indicating dissociation of two abilities. However, a cooperative role of haemadsorbing ability in the initiation of cell fusion can not be excluded. This is compatible with the recent findings that L cell-grown Sendai virus was deficient in haemolytic and cell-fusing activities whereas it possessed haemagglutinating and neuraminidase activities, and trypsin treatment of the virus particle restored preferentially the two activities in the former group (Homma, 1971, 1972; Homma & Tamagawa, 1973). The dissociation of haemolytic and cell-fusing activities from haemagglutinating and neuraminidase activities was suggested on the basis of the polypeptide analysis of Sendai virus by polyacrylamide gel electrophoresis. The defective L cell-grown Sendai virus essentially contained two major glycoproteins, the larger glycoprotein, GP1, and the smaller one, GP2. The trypsin treatment which restored both haemolytic and cell-fusing activities of L cell-grown Sendai virus selectively cleaved GP2 into much smaller glycoproteins. In contrast, egg-grown Sendai virus which exhibited full biological activities only showed the cleaved form of GP2 (Homma & Ohuchi, 1973). It was separately shown that the blocking activity of haemagglutination inhibition and neuraminidase of Sendai virus reside on a single glycoprotein which was in good accordance with GP1 (Tozawa, Watanabe & Ishida, 1973).

In a practical sense, the results from the present experiments suggest a procedure for isolating causative agents from tissues latently infected with paramyxovirus and the related viruses. In case of subacute sclerosing panencephalitis, direct isolation of the agent from the lesion of the brain tissue has failed and succeeded only when the cells from the lesion were co-cultivated with sensitive cells. In such case, however, the authors claimed that dispersion of the tissues by trypsin gave success among several trials (Baublis & Payne, 1968). In view of present results, their trypsin procedure might activate the principle on the cell surface responsible for cell fusion and thus enable the sensitive cells to be infected. Using this procedure, we may facilitate the isolation of unknown viruses in a masked state from latently infected tissues of multiple sclerosis (Meulen et al. 1972; Prineas, 1972), polymyositis (Chou, 1967) and systemic lupus erythematosus (Györkey et al. 1969).

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REFERENCES


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