Persistent Infection of a Cell Line of Mouse Origin after Cell Fusion by u.v.-inactivated Sendai Virus

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SUMMARY

A cell line derived from Sendai virus-induced fusion of human adenocarcinoma and CBA mouse embryo cells had Sendai virus antigen (detected by immunofluorescence), together with bi-armed marker chromosomes, in 100% of the cells. After repeated passage, antigen-free cells carrying the same marker chromosomes appeared in the culture. Acrylamide gel analysis showed that all the Sendai virus antigens of antigen-positive cells were normal with the exception of the M protein. Antigen-negative cells contained no virus proteins and could be superinfected with wild-type virus, when all virus proteins appeared.

INTRODUCTION

Persistent non-productive or poorly productive infection of cell cultures is a characteristic property of the Paramyxovirus group of viruses. The nature of the relationship between the cell and the carried virus is not well understood. An experimental system in which a cell line was persistently infected by a defined defective mutant virus would obviously be of value in the analysis of the phenomenon, but would be difficult to achieve by deliberate experimental design. This paper describes such a cell line, which was obtained by chance in attempts to make hybrids between human tumour and primary mouse cells.

METHODS

Cells. The derivation of the cell line 55ME4 from an experiment in which cells of a continuous line of human adenocarcinoma of the rectum (HT55; Watkins & Sanger, 1977) were fused with primary CBA mouse embryo cells, has been described elsewhere (Watkins, 1977). Chromosome spreads were made by the coverslip method (Watkins, 1971) and Giemsa banding was by the acetic-saline Giemsa method of Sumner et al. (1971).

Virus. The strain of Sendai virus used for fusion was obtained from the Sir William Dunn School of Pathology, Oxford, where it has been used for cell fusion studies since 1964. Stock virus was inactivated by exposure to u.v. light for 30 min as described elsewhere (Watkins, 1971).

Immunofluorescence. Antiserum against Sendai virus was prepared by two injections a week apart of 10^4 haemagglutinating units of infectious virus in 1 ml of saline intravenously into a rabbit which was bled 2 weeks after the second injection. Fluorescein
labelled goat-anti-rabbit-IgG serum was obtained from Nordic Immunochemicals (Maidenhead, U.K.). The sandwich method of labelling acetone-fixed cell cultures on coverslips was carried out by standard methods and has been described elsewhere (Watkins, 1977).

**Infection of cells and metabolic labelling of virus products.** All cells were maintained as confluent monolayers in 90 mm plastic Petri dishes. Cells were washed once with PBS and then infected with Sendai virus at 3 to 10 p.f.u./cell. After adsorption for 60 min at room temperature, the inoculum was removed and the cells washed twice in PBS, then fed with either maintenance medium or labelling medium containing radioactive precursors and incubated at 37 °C.

Metabolic labelling of virus products was accomplished by incubation of cells after virus adsorption with Dulbecco's modification of Eagle's medium (DME) modified to contain 10% of the normal concentration of methionine supplemented with 20 to 50 μCi/ml of 35S-methionine. All media used in labelling experiments contained 2% dialysed foetal calf serum.

Labelling of intracellular virus products was accomplished by addition of labelling medium 16 h after virus adsorption. At varying times thereafter, cells were washed once with PBS and then either disrupted directly by addition of the electrophoresis dissociation buffer or for pulse-chase experiments, put into maintenance medium for further incubation at 37 °C and subsequent disruption as described.

**Preparation and immunoprecipitation of virus and cell extracts.** Samples of Sendai virus to be immunoprecipitated were diluted to 0.1 ml with buffer (0.01 M-tris-HCl, pH 7.0, 0.025 M-KCl, 0.005 M-MgCl2) and made 1% with respect to Triton X-100 and sodium deoxycholate. Cell cultures were prepared by homogenizing the washed cells in 1 ml of buffer containing 1% Triton X-100 and 1% sodium deoxycholate. The nuclei were removed by centrifugation at 1000 g for 5 min and the supernatant clarified by centrifugation at 100,000 g for 40 min.

These samples were immunoprecipitated as described by Kessler (1975). To each sample 5 μl of anti-Sendai serum or control serum was added and incubated at room temperature for 1 h. A 10% suspension of formaldehyde-fixed *Staphylococcus aureus*, Cowan strain A, washed five times in tris-saline (0.05 M-tris-HCl, pH 7.2; 0.15 M-NaCl, 0.005 M-EDTA) was added as an immunoglobulin (Ig) adsorbent for a further hour and incubated at room temperature. The bacterial suspension was then washed three times in buffer and the Ig complexes recovered by addition of 50 μl of electrophoresis dissociation buffer, heated to 100 °C for 1 min. The bacteria were removed by centrifugation for 1 min in a Beckman microfuge, model B, and the supernatant samples loaded directly on to a SDS-polyacrylamide gel. The efficacy of the procedure and the specificity of the anti-Sendai serum were tested using purified disrupted Sendai virus. The anti-Sendai serum efficiently immunoprecipitated all the Sendai structural proteins.

**Polyacrylamide gel electrophoresis.** The immunoprecipitates or whole virion preparations were dissociated and separated on slab gels using the procedure of SDS-polyacrylamide gel electrophoresis described by Laemmli (1970). All samples were taken up in electrophoresis dissociation buffer (final concentration 0.0625 M-tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and were heated to 100 °C for 2 min before loading on to the gel. 20 to 50 μl of each sample was then loaded on to the 2 mm thick by 12 cm long slab gel formed and maintained between parallel glass plates. The stacking gel had a final composition of 5% acrylamide/0.13% bis in 0.25 M-tris-HCl buffer, pH 6.8, containing 0.1% SDS and 1 mg/ml ammonium persulphate. Polymerization was initiated by the addition of TEMED, 1 μl/ml. The composition of the running gel was 10% acrylamide/0.27% bis in 0.4 M-tris-HCl, pH 8.8, containing 0.1% SDS and 1 mg/ml ammonium persulphate. Polymerization was initiated as above. Running buffer consisted of 0.025 M-tris-HCl, 0.192 M-glycine and 0.1% SDS at a final pH of 8.3.
Persistent infection by Sendai virus

Samples were run into the stacking gel for 30 min at 80 V and then run either for approx. 4 h at 120 V or 16 h at 30 V. After electrophoresis the gels were fixed in 10% trichloroacetic acid at 4 °C for 1 h, washed in water for 2 h and dried under vacuum. The labelled proteins were located by autoradiography of the gel using Kodak X-ray film (X-Omat H1).

RESULTS

Characteristics of the cell lines studied

The cell line 55ME4 grew well in 10% foetal calf serum and Eagle's minimal essential medium, and showed marked density-dependent inhibition of growth at confluence. Immunofluorescence tests with a rabbit antiserum against Sendai virus showed strong cytoplasmic fluorescence in 100% of the cells, of the type characteristic of Sendai virus infection. Pre-immunization rabbit serum produced no fluorescence. 100% of the cells also showed strongly positive haemadsorption of sheep erythrocytes (Fig. 1). No tumours developed after subcutaneous injection of 10^6 to 10^7 cells into five athymic mice and five CBA adult mice.

When the line had grown for 31 further passages (55ME4/P31) it was tested again with rabbit anti-Sendai serum. At this time about 40% of the cells showed no fluorescence (Fig. 1). This suggested that virus-negative clones were arising in the population. Haemadsorption-positive and haemadsorption-negative colonies which had developed from cells seeded at low density (100 cells per 25 cm^2 flask) were therefore isolated and examined for Sendai fluorescence. Two subclones with 100% positive cells (55ME4/3 and 55ME4/I) and two subclones with 100% negative cells (55ME4/D and 55ME4/H) were grown for further examination.

The characteristics of the karyotypes of 55ME4 and the five sublines are summarized in Table I. All the sublines were subtetraploid and all except 55ME4/I contained two to nine bi-armed marker chromosomes (Fig. 2). The origin of the bi-armed chromosomes is still under investigation. Some of the bi-armed chromosomes had Giemsa-banding patterns resembling human chromosomes; others did not and from their size relative to the largest mouse chromosomes were considered to have arisen by centric fusion of mouse chromosomes. For the purpose of the present paper the question of the hybrid nature of these cells is not relevant and will be discussed elsewhere. Other lines, not persistently infected with Sendai virus, which had been derived from the original fusion experiment and from fusion experiments with other human tumour lines, did not contain bi-armed chromosomes (Watkins, 1977). It was therefore concluded that the presence of bi-armed marker chromosomes in 55ME4 and the sublines recovered from it proved that the Sendai-negative sublines must have arisen from Sendai-positive parental cells and that they were not contaminating cells which had increased in proportion as the culture grew. This conclusion was strengthened by the similarity of the Giemsa banding patterns of the bi-armed chromosomes in 55ME4 and the derived sublines.

Superinfection of 55ME4/H with Sendai virus

Cultures of confluent 55ME4/H (Sendai-negative) on 16 mm coverslips were overlaid with 20 μl of infectious Sendai virus stock and incubated for 30 min at room temperature in 60 mm plastic Petri dishes. Five ml of medium were then added and incubation continued at 37 °C. Control cells were mock-infected with medium alone and with stock Sendai virus which had been irradiated with u.v. light for 30 min. Immunofluorescence 3 days later with rabbit anti-Sendai serum showed that the control coverslips contained no positive cells, while about 4% of the cells given infectious virus were positive. All the foci of infection consisted of single cells only. Immunofluorescence after a further 3 days showed no
Fig. 1. (a) Five cells of subclone 55ME4/P31 stained with rabbit-v-Sendai serum and fluorescein-labelled Goat-v-rabbit IgG. Three cells show strong fluorescence; two are negative. (b) Cells of 55ME4 showing haemadsorption. (Coverslip cultures were kept for 30 min at 4 °C in 1 % sheep erythrocytes in saline, washed, fixed and stained with Giemsa stain.)

Table 1. Karyotypes of Sendai-positive and Sendai-negative cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sendai (%)</th>
<th>Chromosome number</th>
<th>Cells with bi-armed chromosomes (%)</th>
<th>Bi-armed chromosomes/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>55ME4</td>
<td>100</td>
<td>64-83</td>
<td>73-82</td>
<td>77±5</td>
</tr>
<tr>
<td>55ME4/P31</td>
<td>60</td>
<td>69-84</td>
<td>73-81</td>
<td>77±2</td>
</tr>
<tr>
<td>55ME4/3</td>
<td>100</td>
<td>62-80</td>
<td>70-75</td>
<td>71±6</td>
</tr>
<tr>
<td>55ME4/I</td>
<td>100</td>
<td>57-78</td>
<td>66-74</td>
<td>69±1</td>
</tr>
<tr>
<td>55ME4/D</td>
<td>0</td>
<td>70-78</td>
<td>76-77</td>
<td>74±1</td>
</tr>
<tr>
<td>55ME4/H</td>
<td>0</td>
<td>60-85</td>
<td>68-76</td>
<td>70±2</td>
</tr>
</tbody>
</table>

significant increase in the number of foci of fluorescent cells, but 70 % of the foci consisted of two to six positive cells. Control coverslips continued to be negative. From this experiment it was concluded that 55ME4/H cells were susceptible to infection by Sendai virus and were capable of producing infectious progeny virus.

Characterization of Sendai virus specific intracellular proteins

Immunofluorescent examination of the various cell lines with antiserum against Sendai virus suggested that Sendai virus-specific proteins were being synthesized only in the 55ME4, 55ME4/3 and 55ME/I cell lines and that no detectable virus products were present in the 55ME4/D and 55ME4/H cells. To confirm these observations, cultures of both
positive and negative cells were metabolically labelled with $^{35}$S-methionine, and cytoplasmic extracts prepared for immunoprecipitation and subsequent examination in a Laemmli SDS-polyacrylamide gel system as described. The results of these investigations are demonstrated in Fig. 3. No virus-specific products could be identified in the immunofluorescent-negative cell line 55ME4/D even after long periods of exposure to $^{35}$S-methionine. When 55ME4/D cells were infected with the original strain of Sendai virus, cellular extracts gave a series of bands migrating in a similar way to those found in Sendai virus-infected cells by others (e.g. Yoshida et al. 1979; Fig. 3, CI). Similar bands were identified in lysates of purified Sendai virus; these latter were too faint to allow adequate reproduction and so are not included in Fig. 3. Extracts of 55ME4/3 (antigen-positive) cells produced a series of bands which were identical to those from infected 55ME4/D cells. However, only one band was present in the M region of the former, while the latter showed two. The single M region band from 55ME4/3 cells corresponds to the slower moving of the two M region
bands from infected 55ME4/D cells. The apparent mol. wt. of this single band was 36,000, which is similar to that of the M protein of a recently reported mutant of Sendai virus (Yoshida et al. 1979) and to the protein, designated 'B' by Lamb & Choppin (1977), which is thought to be a phosphorylated form of the M protein.

To determine whether the missing band in the 55ME4/3 cells could be due to the production of an unstable protein that was either rapidly degraded or cleaved, 55ME4/3 cells were pulsed for 10 min with 35S-methionine, then either lysed directly or after varying intervals of incubation in complete medium and cellular extracts examined for a band having faster mobility. No such band could be demonstrated, even in cultures fixed immediately after labelling. Incorporation of radiolabel was observed, however, in the band having slower electrophoretic mobility and under chase conditions no new bands were detected.
DISCUSSION

The results of the polyacrylamide gel examination of the Sendai virus-specific intracellular proteins synthesized in the 55ME4/3 cells suggest an abnormality in either the synthesis or metabolism of Sendai virus M protein. From the data available, it is impossible to exclude the possibility that a normal protein is synthesized, which is rapidly degraded or converted to a different form, either by cleavage or phosphorylation. The fact that no precursor-product relationship could be established by pulse-chase experiments, however, makes this possibility highly unlikely. Furthermore, the observation of normal M region bands after Sendai virus infection of the clonally related 55ME4/D and H cells argues against specific cellular modification of this virus protein.

These results are best explained by the virus directed synthesis of an abnormal M protein demonstrating altered mobility on polyacrylamide gel analysis. The derivation of these cell lines suggests the generation of a Sendai virus mutant that, under these conditions of tissue culture, was able to establish and maintain a persistent, non-lytic infection. The role of an abnormal M protein in this process is not clear, but abnormalities in M protein have been associated with persistent infections not only with Sendai virus but also with measles-like viruses isolated from subacute sclerosing panencephalitis (Schluederberg et al. 1974; Wechsler & Fields, 1978). The significance of these observations is not known but is believed to relate to the requirement of a normal M protein for virus maturation and release. Further studies with the 55ME4/3 cells suggest that these processes are substantially altered in this cell line as no infectious virus can be shown to be released (T. J. Schnitzer et al., unpublished observations).

The mechanism by which this persistent infection arose is obscure. Inactivated Sendai virus has been used for the production of cell hybrids for many years in many laboratories, but persistent infection of hybrid lines has not so far been reported, possibly because it has not been sought. Because of the complications that persistent infection could introduce into hybrid cell experiments, especially those concerned with cellular antigens or tumorigenicity, routine screening by immunofluorescence of Sendai-produced hybrids would be a wise precaution. Another curious feature was the presence of bi-armed marker chromosomes in this line and their absence from other, Sendai-negative, lines produced in the original fusion experiment. It may be that persistent infection promotes centric fusion. This could cause misleading results in chromosomal analysis of putative human × mouse hybrid lines if they are persistently infected. The persistence of bi-armed chromosome markers was not absolutely correlated with persistent infection, since they were absent from 55ME4/I cells, which contained Sendai virus antigens (Table I).

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REFERENCES


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