INTERSPECIFIC FUSION OF BOVINE AND OTHER CELLS WITH PARAINFLUENZA VIRUSES (SENDAI AND Pi-3)

By

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INTRODUCTION

The artificial fusion of cells of different type and species, for the formation of heterokaryons and hybrid cells, is now widely used, since Harris and Watkins (1965) demonstrated the practicality of this technique, with Sendai virus as a fusing agent.

A variety of viruses show fusing properties (Poste, 1972), but in most artificial systems Sendai virus (parainfluenza virus type 1) has been used, because of the wide range of cells which contain surface receptor sites for it (Harris, 1970). However, cells do vary in their fusing response to Sendai virus (Okada and Tadokoro, 1963), and Irvin, Brown, Stagg, Kanhai and Rowe (1975a) and Irvin, Stagg and Kanhai (1976) obtained disappointing results with Sendai virus in bovine/hamster and bovine/mouse fusion systems in that fusion percentage was low and no hybrid lines were developed. Consequently it was decided to investigate the use of a virus with more specific affinity for bovine cells as an alternative fusing agent.

Omar (1965) showed that bovine parainfluenza virus type 3 (Pi-3) caused extensive intraspecific fusion of bovine and other cells in vitro and Kita and Gillespie (1968) examined the susceptibility of various cell types to bovine Pi-3 virus, noting fusion in a number of cases. The current study compares the fusing properties of Sendai and Pi-3 in a variety of interspecific cell systems with particular emphasis on bovine cells.

MATERIALS AND METHODS

Viruses

Sendai virus was grown in embryonated hens' eggs by the method described by Watkins (1971).

Pi-3 virus (J121 strain, Betts, Jennings, Omar, Page, Spence and Walker, 1964) was grown on calf kidney monolayer cells as described by Omar (1965).

The same 2 batches of Sendai and Pi-3 viruses were used throughout and were stored in gas-phase liquid nitrogen.

Prior to fusion experiments, aliquots of virus were thawed in a 37 °C waterbath, inactivated for 30 min with ultraviolet (u.v.) light and assayed for haemagglutinating units (HAU) against human (group O) erythrocytes (Harris, Watkins, Ford and Schoefl, 1966). Virus suspensions were then diluted with ice-cold Hanks balanced salt solution (HBSS). Inactivation was confirmed by failure of virus to infect secondary calf kidney monolayer cells.
The following cells were used:

**Bovine cells.** (1) Bovine lymphocytes were collected by cannulation of a prefemoral efferent lymphatic duct of a normal calf. (2) Bovine erythrocytes were obtained from jugular vein blood of a normal calf and collected into Anticlot (Clinton Laboratories, Santa Monica, California, U.S.A.). (3) The MDBK line of bovine kidney cells (Madin and Darby, 1958). (4) The E174 culture of *Theileria parva*-infected bovine lymphoid cells. This culture is similar to those described by Malmquist, Nyindo and Brown (1970) and was grown by the same methods.

**Mouse cells.** (1) Mouse A9 cells (Littlefield, 1964) are derived from L cells (Earle, 1943) and lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), consequently they are unable to utilize exogenous sources of hypoxanthine and therefore unable to grow in hypoxanthine/aminopterin/thymidine (HAT) medium (Szybalska and Szybalski, 1962) in which de novo purine synthesis is blocked by aminopterin. (2) B82 cells (Littlefield, 1966) are also derived from L cells but lack the enzyme thymidine kinase (TK); this also prevents their growth in HAT medium since they cannot utilize exogenous thymidine, de novo pyrimidine synthesis being blocked by aminopterin.

**Human cells.** The S3 strain of HeLa cells (Puck, Marcus and Cieciura, 1956).

**Pig cells.** Pig thyroid (PT) cells were from a line established at this Institute (Rutter and Luther, 1973).

**Avian cells.** Erythrocytes were obtained from 12-day domestic hen embryos by cutting the main blood vessels, collecting the fluid into phosphate-buffered saline (PBS) and centrifuging out the erythrocytes.

**Maintenance of Cell Lines**

Cell lines were maintained in Eagle's Minimal Essential Medium with Earle's salts supplemented with 10 per cent foetal calf serum, 100 iu/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml mycostatin (basic medium). Slight variations of this basic medium were used for some lines; these details are given in the relevant papers. Hybrid cells were selected, in some experiments, by growth in HAT medium (basic medium supplemented with $1 \times 10^{-5}$ M hypoxanthine, $4 \times 10^{-7}$ M aminopterin, $1.6 \times 10^{-5}$ M thymidine, $3 \times 10^{-6}$ M glycine and $1 \times 10^{-2}$ M sodium pyruvate).

**Harvesting of Cells**

Cells were washed and harvested by standard techniques. Erythrocytes were counted on a Coulter Counter (Coulter Electronics, Harpenden, Hertfordshire) and all other cells in a counting chamber (Improved Neubauer, Gelman, Hawksley, Lancing, Sussex). Cells were then diluted in HBSS to give appropriate concentrations for fusion.

**Fusion Procedures**

Two fusion techniques were used: (a) Suspension fusion techniques were based on those described by Harris *et al.* (1966) and by Watkins (1971). Cells in suspension were mixed at appropriate concentrations and after fusion with virus, aliquots of cell/virus suspensions were dispensed into 6-cm diameter Petri dishes containing coverslips. The dishes were incubated at 37°C in a closed cabinet containing 5 per cent CO₂ in air. (b) Monolayer fusion techniques were based on those described by Carlson, Savage and Ringertz (1970) and Irvin *et al.* (1975a). Twenty-four hours prior to fusion, aliquots of approximately $5 \times 10^{8}$ monolayer-forming cells were pipetted into 6-cm Petri dishes containing coverslips. Petri dishes were then incubated as above. For fusion the medium was pipetted off and the monolayers overlaid with a suspension of the relevant virus and cell mixture. After fusion the
supernatant was pipetted off and replaced with basic medium. The Petri dishes were incubated as before.

Autoradiography

In some experiments, one group of cells was pre-labelled with tritiated \(^{3}\text{H}\) thymidine, by the addition, 24 h before fusion, of 0.25 mCi of \([^{3}\text{H}]\)thymidine per ml of culture \((6-^{3}\text{H})\)thymidine, Radiochemical Centre, Amersham, Bucks). After fusion, coverslips were washed and examined by autoradiography, as previously described (Irvin et al., 1975a), following a 5-day exposure period.

Examination of Cultures

Giesma-stained coverslips were prepared 24 h after fusion and examined as described by Irvin et al. (1975a). The following parameters were determined: (a) Percentage of polykaryons or multinucleate cells (on counts of 200 cells). (b) Mean number of nuclei in polykaryons (on counts of 50 polykaryons). When heterokaryons could be easily distinguished (e.g. in HeLa/chick erythrocyte systems) the mean number of the different nuclei was recorded separately. (c) Percentage of heterokaryons (on counts of 100 polykaryons). In cases where heterokaryons and homokaryons were morphologically indistinguishable (e.g. in A9/MDBK systems), parameter (c) was determined on autoradiography. The relative numbers of nuclei in such heterokaryons was also recorded following autoradiography.

RESULTS

HeLa/Chick Erythrocyte Fusion

Aliquots of \(1 \times 10^6\) HeLa cells and \(5 \times 10^7\) chick erythrocytes were mixed for suspension fusion with u.v. light inactivated Sendai and Pi-3 viruses and with non-inactivated Pi-3 virus, as follows: (1–2) Sendai virus (inactivated) 3000 HAU and 300 HAU, (3–4) Pi-3 virus (inactivated) 3000 HAU and 300 HAU, (5–6) Pi-3 virus (non-inactivated) 3000 HAU and 300 HAU and (7) Control with no virus.

<table>
<thead>
<tr>
<th>Virus and HAU/ml</th>
<th>Per cent polykaryons</th>
<th>Mean number of nuclei per polykaryon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterokaryons</td>
<td>HeLa homokaryons</td>
</tr>
<tr>
<td>Sendai (3000)</td>
<td>69.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Sendai (300)</td>
<td>64.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Pi-3 active (3000)</td>
<td>10.0</td>
<td>59.0</td>
</tr>
<tr>
<td>Pi-3 active (300)</td>
<td>4.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Pi-3 inactivated (3000)</td>
<td>3.5</td>
<td>33.0</td>
</tr>
<tr>
<td>Pi-3 inactivated (300)</td>
<td>2.5</td>
<td>21.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Giesma-stained coverslips were examined after 24 h. The results are shown in Table 1. A high percentage of heterokaryons was formed with Sendai virus.
(Fig. 1), but with Pi-3 virus the chick erythrocytes apparently possessed few virus receptor sites and the percentage of heterokaryons was low (Fig. 2). There was some reduction in fusing activity of inactivated Pi-3 virus compared with non-inactivated virus.

![Fig. 1. Chick/HeLa heterokaryons formed by Sendai virus fusion. The small chick erythrocyte nuclei can be readily differentiated from the much larger HeLa cell nuclei. Giemsa. ×936.](image1)

![Fig. 2. HeLa cell homokaryons formed by Pi-3 virus fusion. No heterokaryons are present and 2 chick erythrocytes have failed to fuse and show no evidence of haemolysis. Giemsa. ×936.](image2)

HeLa cells fused readily to each other in the presence of Pi-3 virus and homokaryons with large numbers of nuclei were commonly seen (Fig. 2). The mean number of nuclei in HeLa homokaryons formed by fusion with Pi-3 virus was higher than with Sendai virus.

**A9/B82 Fusion**

Aliquots of $2.5 \times 10^6$ A9 cells were mixed with $2.5 \times 10^6$ B82 cells. Suspension fusions were carried out using 4000 HAU of inactivated Pi-3 virus or 4000
HAU of inactivated Sendai virus. Controls without virus were also set up. After fusion, cells were transferred to Petri dishes and maintained for 24 h in basic medium. Basic medium was then replaced by HAT medium in all Petri dishes. HAT medium was changed after 2, 6, 13 and 16 days. The number of colonies of A9/B82 hybrids was counted after 19 days.

The percentage of multinucleate cells formed after 24 h and the mean number of nuclei in such cells are shown in Table 2. The fusion percentage was similar for both viruses, but the mean number of nuclei was marginally higher in cells fused with Pi-3 virus. Heterokaryons and homokaryons were not differentiated. Table 2 also shows the number of colonies of hybrid cells which grew in HAT medium. More hybrids resulted from Sendai virus fusion than from Pi-3 virus.

**A9 Fusion with MDBK and PT Cells**

Aliquots of $5 \times 10^6$ A9 cells were mixed with similar aliquots of MDBK and PT cells for suspension fusion with 4000 HAU of Pi-3 and Sendai viruses.

### Table 2

<table>
<thead>
<tr>
<th>Cell/virus mixture</th>
<th>Per cent polykaryons</th>
<th>Mean number of nuclei per polykaryon</th>
<th>Number of hybrid colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9/B82 (Sendai)</td>
<td>52.5</td>
<td>3.4</td>
<td>18</td>
</tr>
<tr>
<td>A9/B82 (Pi-3)</td>
<td>54.0</td>
<td>4.4</td>
<td>3</td>
</tr>
<tr>
<td>A9/B82 (No virus)</td>
<td>2.0</td>
<td>2.4</td>
<td>1</td>
</tr>
<tr>
<td>A9</td>
<td>2.5</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>B82</td>
<td>0.5</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of colonies from 3 Petri dishes for each cell group (seeding rate per dish was $1 \times 10^6$ cells).

### Table 3

<table>
<thead>
<tr>
<th>Cells and virus mixture</th>
<th>Per cent polykaryons</th>
<th>Mean number of nuclei per heterokaryon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterokaryons</td>
<td>Total</td>
</tr>
<tr>
<td>A9/PT (Pi-3)</td>
<td>13.6</td>
<td>40.0</td>
</tr>
<tr>
<td>A9/PT (Sendai)</td>
<td>13.1</td>
<td>30.5</td>
</tr>
<tr>
<td>A9/MDBK (Pi-3)</td>
<td>20.9</td>
<td>43.5</td>
</tr>
<tr>
<td>A9/MDBK (Sendai)</td>
<td>2.2</td>
<td>11.5</td>
</tr>
<tr>
<td>A9</td>
<td>—</td>
<td>3.5</td>
</tr>
<tr>
<td>APT</td>
<td>—</td>
<td>5.0</td>
</tr>
<tr>
<td>MDBK</td>
<td>—</td>
<td>4.0</td>
</tr>
</tbody>
</table>
The A9 cells were pre-labelled by 24 h exposure to [³H]thymidine prior to fusion. Autoradiography showed that 85 per cent of A9 cells incorporated the isotope.

Fusion percentages were determined after 24 h; homokaryons and heterokaryons being differentiated on autoradiography. The results are shown in Table 3. Fusion of A9 cells with PT cells was similar with both Pi-3 and Sendai viruses, but the percentage of fusion of A9 cells with MDBK cells was greater with Pi-3 than with Sendai virus (Figs 3 and 4). Autoradiography

confirmed that MDBK cells had a much greater affinity for Pi-3 virus than for Sendai since bovine nuclei predominated in heterokaryons formed by Pi-3 virus fusion (Fig. 5), whereas the numbers of mouse and bovine nuclei were similar in Sendai virus-induced heterokaryons.
A9 Fusion with Bovine Erythrocytes

Aliquots of $5 \times 10^5$ A9 cells were seeded in Petri dishes containing coverslips. Monolayer fusion was carried out with $2 \times 10^7$ bovine erythrocytes with Sendai and Pi-3 viruses. Coverslips were stained and examined after fusion at time 0 (immediately prior to warming to 37 °C) and then after $\frac{1}{2}$, 2 and 24 h.

At time 0 the majority of A9 cells had bovine erythrocytes adherent to their surface. In the Pi-3 virus preparation the value was 80.5 per cent and in the Sendai virus preparation 71.0 per cent. The mean number of erythrocytes adhering to each A9 cell was 4.2 in the Pi-3 virus preparations and 3.5 in the Sendai virus preparations.

As soon as cell suspensions were warmed to 37 °C there was rapid haemolysis and intact erythrocytes were rare in the $\frac{1}{2}$-h preparations and absent from the others. Intraspecific fusion of A9 cells occurred readily as noted previously.

B82 Fusion with E174 Cells

Aliquots of $2 \times 10^5$ B82 cells were seeded into Petri dishes containing coverslips and after 24 h monolayer fusion was carried out with $2 \times 10^6$ E174 cells per dish and 2 concentrations each of Pi-3 and Sendai viruses. Coverslips were examined after 24 h and the percentage of heterokaryons determined. Such
cells were detected on morphological differentiation of nuclei and by the presence of macroschizonts of *T. parva* (Fig. 6).

The results are shown in Table 4. Fusion percentage was generally low and heterokaryons were commonly seen only in the preparation treated with 1000 HAU of Pi-3 virus.

**TABLE 4**

<table>
<thead>
<tr>
<th>Virus concentration (HAU)</th>
<th>Heterokaryons</th>
<th>B82 homokaryons</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi-3 (100)</td>
<td>0.4</td>
<td>7.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Pi-3 (1000)</td>
<td>7.0</td>
<td>22.0</td>
<td>29.0</td>
</tr>
<tr>
<td>Sendai (100)</td>
<td>0.5</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Sendai (1000)</td>
<td>0.5</td>
<td>7.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Control (no virus)</td>
<td>1 only seen</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Cultures were maintained in HAT medium for a further 6 weeks but no hybrids lines were isolated.

**A9 Fusion with Bovine Lymphocytes**

Aliquots of $2 \times 10^8$ A9 cells were mixed with $2 \times 10^7$ bovine lymphocytes for suspension fusion with 2 concentrations each of Sendai and Pi-3 virus. Coverslips were examined after 24 h and the results are shown in Table 5.

![Fig. 7. A9/bovine lymphocyte heterokaryon. The denser lymphocyte nuclei are easily distinguished. Giemsa. ×936.](image)

The majority of heterokaryons were seen in preparations treated with Pi-3 virus (Fig. 7). In that treated with 2000 HAU of Pi-3 virus the mean number of nuclei was 2.1 bovine and 1.7 A9; whereas in the 2000 HAU Sendai virus preparation the numbers were 1.0 bovine and 1.5 A9 nuclei.
TABLE 5

FUSION OF A9 CELLS AND BOVINE LYMPHOCYTES WITH 2 CONCENTRATIONS EACH OF INACTIVATED SENDAI AND PI-3 VIRUSES: CELL COUNTS 24 H AFTER FUSION

<table>
<thead>
<tr>
<th>Virus concentration (HAU)</th>
<th>Per cent polyparyons</th>
<th>Heterokaryons</th>
<th>A9 homokaryons</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi-3 (200)</td>
<td>19.1</td>
<td>16.9</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>Pi-3 (2000)</td>
<td>25.5</td>
<td>12.0</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>SV (200)</td>
<td>0.4</td>
<td>2.6</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>SV (2000)</td>
<td>2.9</td>
<td>5.6</td>
<td>8.5</td>
<td></td>
</tr>
</tbody>
</table>

Cultures could not be maintained in HAT medium and no hybrid lines were isolated.

DISCUSSION

A wide variety of cell types contain receptor sites for Sendai virus which has therefore been used in most cell fusion procedures. The current work shows however, that a bovine strain of Pi-3 virus can also fuse cells from a variety of species including mouse, man, pig and cattle. Only chick erythrocytes were apparently refractory to Pi-3 virus fusion. In the case of cattle cells, superior fusion was achieved with Pi-3 virus. This finding probably relates to the presence of bovine host cell constituents in the Pi-3 viral envelope (Kates, Allison, Tyrell and James, 1961), thus explaining Pi-3's greater affinity for bovine cells, compared with that of chick-derived Sendai virus. Further work will be necessary to determine whether this finding will have practical application in the development of bovine hybrid cell lines. The possibility arises, however, that cells from other species may be more amenable to fusion if the virus is grown in cells of that species. For example, most strains of Sendai virus currently used for fusion are grown in embryonated chicken eggs but, although such strains generally fuse a wide spectrum of cell types, improved fusion may be achieved in some situations by the use of specific species-derived strains.

The main purpose of the current work was to find a fusing agent more suitable than Sendai virus for the fusion of bovine cells. Bovine Pi-3 virus was chosen, and in addition to examining its ability to fuse bovine cells, a number of other cell systems was examined. The HeLa/chick erythrocyte system represents fusion of cells of different classes and Sendai virus fusion of these cells has been well documented by Harris (1965) and Harris et al. (1966).

The spontaneous fusion of enzyme-deficient A9 and B82 mouse cells has been described by Littlefield (1964), and a detailed study of Sendai virus-induced hybrids is given by Engel, McGee and Harris (1969).

The fusion of A9 cells with PT cells was previously untried. They were chosen partly for this reason and partly to provide a further contrast of the fusing ability of cells of different species.

In these 3 systems, the intra- and interspecific fusing abilities of Sendai and Pi-3 virus were similar, except that chick erythrocytes could not be readily
fused by Pi-3. A further difference noted was that cells clumped together more rapidly when mixed in the presence of Sendai virus than with Pi-3. Despite this, the final number of nuclei in Pi-3-induced polykaryons was usually higher than that in polykaryons induced by Sendai virus.

In all but the first experiment, inactivated Pi-3 virus was used, since inactivation did not appreciably alter the fusing ability of the virus. This removed any complicating factors which might have been introduced by the use of infectious virus. Harris et al. (1966) also noted only slight loss of fusing capacity when Sendai virus was inactivated.

In the experiments in which bovine cells were used, four widely divergent types were chosen. MDBK cells were from a well-characterized and stable line and could therefore be expected to fuse well. Lymphocytes are difficult to fuse (Harris et al., 1966) and will not normally divide in vitro. They can however, be used to complement enzyme defective cells and resultant hybrids can then be selected in HAT medium (Miggiano, Nabholz and Bodmer, 1969). Transformed bovine lymphoid cells parasitized with T. parva are similarly difficult to fuse (Irvin et al., 1975a); they contain reduced HGPRT activity (Irvin and Stagg, 1977) and heterokaryons contain marker parasites. Their fusion capacity is therefore readily amenable to study.

The fusing ability of bovine erythrocytes was studied as a preliminary to following up earlier work on the fusing ability of Babesia-infected erythrocytes (Irvin, Stagg, Kanhai, Brown and Omwoyo, 1975b).

All 4 types of bovine cell could be fused by both Sendai and Pi-3 virus, but in all cases, with comparable doses of virus, Pi-3 virus produced a higher mean nuclear number.

Limited attempts to isolate hybrid cells were unsuccessful, although such hybrids have been isolated previously (Teplitz, Gustafson and Pellett, 1968).

The physical factors resulting in optimal fusion have been studied by many workers (reviewed by Poste, 1972), and of particular importance are the relative cell numbers used and the titre of virus. These two aspects are the subject of further study with Pi-3 virus, but the results do suggest that this virus may be of value in the fusion of bovine cells and the development of hybrids.

**SUMMARY**

The ability of a chick strain of Sendai virus and of a bovine strain of para-influenza virus type 3 (Pi-3) to fuse cells of different species, was studied in a series of experiments with human (HeLa), mouse (A9 and B82), pig (PT), chick (erythrocytes) and bovine (MDKB, lymphocytes, erythrocytes and Theileria parva-infected lymphoblasts) cells. A variety of cell combinations was tested and intra- and interspecific polykaryons were produced by both viruses. The 2 viruses gave similar fusion percentages in human, mouse and pig systems, but in systems in which bovine cells were used Pi-3 virus consistently gave a higher percentage of polykaryons and a higher mean nuclear number per polykaryon.

The fusing activity of Pi-3 was only slightly reduced following inactivation by u.v. light.
Previous attempts to fuse bovine cells with Sendai virus have given poor results. The current work shows that a bovine strain of Pi-3 virus fuses such cells better than chick-derived Sendai virus. This finding probably relates to the presence of bovine host cell constituents in the Pi-3 viral envelope, thus explaining the affinity of this strain of Pi-3 virus for bovine cells.

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