**A novel method for the detection
of early events in cell-cell fusion
of Semliki Forest virus infected cells
growing in monolayer cultures**

Brief Report

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Summary

Semliki Forest virus infected *Aedes albopictus* cells were used to investigate virus induced cell-cell fusion. It was shown by a novel method that cell-cell fusion was completed within approximately 5 minutes after triggering the fusion event by low pH. This method consists of fixing fusing cells with glutaraldehyde and microinjecting the highly fluorescent and rapidly diffusing dye Lucifer yellow. In contrast, polykaryon formation, the usually used criterion to measure cell-cell fusion occurred only within 30 minutes. Furthermore, it was shown that potassium cyanide, a potent inhibitor of polykaryon formation in the described system, inhibits an early step of membrane-membrane fusion of neighbouring cells.

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Fusion of cell membranes is one of the most dramatic forms of membrane-membrane interactions (10). This fusion event is a characteristic feature of infections with enveloped viruses (1, 2 and 11). Several enveloped viruses have been reported to cause fusion under certain conditions (7, 9 and 13). We have previously described such a system consisting of Semliki Forest virus (SFV; an enveloped, positive strand RNA virus) infected *Aedes albopictus* cells (5). These SFV infected cells undergo cell-cell fusion at mildly acidic pH, leading to polykaryocytes. This process can be inhibited by a wide variety of chemicals (5, 6), which are useful tools for the elucidation of the molecular mechanisms underlying SFV induced cell-cell fusion.

In contrast to fusion events occurring between viruses and artificial membranes, and viruses and biological membranes, onset of cell-cell fusion

of cells growing in monolayer cultures is difficult to detect and to quantitate. The most commonly used method is phase contrast microscopy. However, it does not allow visualization of the initial event of cell-cell fusion. Only the result in form of polykaryons can be clearly observed after approximately 15 minutes. This handicap makes it very difficult to correlate biochemical processes with the progress of the actual fusion event. However, such correlations are mandatory for the elucidation of the fusion mechanism.

In the following we introduce a novel method to monitor cell-cell fusion. Furthermore, we show that potassium cyanide, a potent inhibitor of SFV induced polykaryon formation (4) acts at an early stage in the fusion process.

The aim of this investigation was to clarify the early events in cell-cell fusion and the action of potassium cyanide an inhibitor of oxidative phosphorylation—which inhibits SFV-induced polykaryon formation [4]—on the fusion process. To this end *Aedes albopictus* cells [clone C 6/36, (3)] were infected with SFV. At 16 hours post infection (pi) the growth medium (Mitsuhashi-Maramorosch medium, MM-medium) was exchanged against MM-medium of pH 6. At different times after lowering the pH (0 to 30 minutes) potassium cyanide was added at a final concentration of 1 mM. The formation of polykaryocytes was examined 30 minutes after lowering the pH to 6. The 30 minutes correspond to the normal time required for SFV-induced

Table 1. Fusion of SFV-infected *Aedes* cells in presence of 1 mM KCN

Time of addition of KCN after pH 6 exposure (minutes)	Fusion
0	—
1	—
2	—
3	—
4	—
5	+
10	+++
15	++++
20	++++
30	++++

The pH of the medium of SFV infected *Aedes* cells was lowered to pH 6 at 16 hours p.i. 1 mM KCN (final concentration) was added at the different times indicated above after lowering the pH. Fusion was examined 30 minutes after lowering the pH by means of phase contrast microscopy. The degree of fused cells was assessed as follows: (—) 0–5 per cent, (+) 5–30 per cent, (++) 30–60 per cent, (+++) 60–90 per cent and (++++) 90–100 per cent of the cells were integrated into polykaryocytes, respectively

Table 2. Fusion of SFV-infected *Aedes* cells as a function of time after lowering the pH to 6

Time after exposure to pH 6 (minutes)	Fusion
0	—
1	—
2	—
3	—
4	—
5	—
10	+
15	++
20	+++
30	++++

Aedes cells were infected with SFV. At 16 hours p.i. the growth medium was exchanged against MM-medium of pH 6. Fusion was observed at the times indicated above by means of phase contrast microscopy. The proportion of cells incorporated into polykaryocytes is indicated as described in Table 1

polykaryon formation to be clearly visible in the light microscope (6). As shown in Table 1 potassium cyanide inhibited the fusion process only when added during the first 4 minutes. This result was compared with the time course of SFV-induced fusion which is summarized in Table 2. As it can be seen, the first clear cell-cell fusions were observed after approximately 10 minutes, when 5 to 30 per cent of the cells appeared to be integrated into polykaryocytes. Thus, these results indicate that potassium cyanide inhibits polykaryocyte formation at an early stage. However, the results presented so far do not show whether potassium cyanide inhibits the membrane-membrane fusion or the reorganization of the cellular membranes and the cytoplasm required to form a polykaryon.

To clarify this uncertainty *Aedes* cells were infected with SFV and 16 hours pi the cells were exposed for various times to pH 6. Then, the cells were fixed with 2.5 per cent glutaraldehyde for 30 to 60 minutes at 4°C in isotonic phosphate buffered saline (PBS) at timely intervals after lowering the pH to 6. To remove excess glutaraldehyde the fixed cells were incubated at room temperature for 10 minutes in PBS containing 10 mM lysine. Then the cells were washed three times with PBS. Thereafter, single cells were microinjected with a 5 per cent aqueous solution of Lucifer yellow by means of the glasscapillary microinjection procedure using an Eppendorf microinjector 5242. Distribution of the dye within the injected and neighbouring cells was observed with a Nikon Diaphot fluorescence microscope. Photographs were taken using a 400 ASA Ilford film. Lucifer yellow, a highly fluorescent dye, was reported to spread quickly throughout the injected cell

without crossing the cell membrane (12). Most importantly, the dye frequently spreads *in vivo* from the injected cell to nearby cells. This movement of dye from cell to cell has been termed dye coupling. Dye coupling offers a method of recognizing functional connections between cells by morphological means (12). Thus it could be reasonably assumed that microscopic membrane-membrane fusions—still invisible by phase contrast microscopy—should allow the dye to diffuse from the injected cell into neighbouring cells. That this assumption holds true is depicted in Fig. 1 A where a single, SFV infected cell was injected with Lucifer yellow 5 minutes after exposure to pH 6. It can be clearly seen that three cells had fused by this time. Even discrete fusions could be detected as indicated by the arrows. With the phase contrast microscope it was impossible or questionable to come to the same conclusion (Fig. 1 B). The same experiment was carried out at different times after lowering the pH to 6. In each experiment 50 to 100 arbitrarily chosen, single cells were injected with the dye. The spreading into neighbouring cells was recorded. The degree of fusion was expressed as percentage of cells showing dye coupling versus the total number of fluorescent cells. This is depicted as a function of time in Fig. 2. Some degree of dye coupling was also detected at time zero of the exposure to pH 6. At time zero the average number of cells involved in a dye coupling event was 2.4 ± 0.8 . This background could reflect dividing cells or other naturally occurring connections between

Fig. 1. SFV-infected cells were exposed at 16 hours p.i. for 5 minutes to MM-medium of pH 6. Then the cells were fixed with glutaraldehyde and processed as described in the text. Single cells were then microinjected with Lucifer yellow and the spreading of the dye into neighbouring cells was monitored with a fluorescence microscope. Three fused cells are depicted in **A** with the arrows indicating microscopic membrane-membrane fusions which can not be identified as clear fusions in the phase contrast microscope (**B**). The asterisk marks the injected cell

Fig. 2. SFV-infected cells were exposed for various times at 16 hours p.i. to medium of pH 6 either in presence or absence of 1 mM KCN. Then the cells were fixed and microinjected with Lucifer yellow. The extent of fusion was expressed as percentage of cells showing dye coupling versus the total of cells containing Lucifer yellow. The inset is showing the average number of cells per dye coupling event (y) versus time (x). ● SFV-infected cells, no KCN; ■ SFV-infected cells, 1 mM KCN

cells. The number of cells in a "coupling" group significantly increased with time after exposure of the infected cells to pH 6 (Fig. 2, inset), thus showing that fusion occurred and progressed. In dye coupling, 90 to 100 per cent of cells involved in fusion was reached after approximately 5 minutes when compared to 30 minutes as judged by phase contrast microscopy. The data presented clearly show that microinjection of Lucifer yellow is a useful tool to monitor cell-cell fusion at its earliest stage.

In similar experiments 1 mM potassium cyanide was added concomitantly with the change of the pH from 7 to 6. This treatment abolished the fusion process. Even when the cells were incubated for 15 minutes in presence of potassium cyanide and medium of pH 6 the degree of cell-cell fusion remained at the control level as shown in Fig. 2. Also the average cell number per dye coupling event (2.1 ± 0.3) did not exceed the value measured for the zero time point in absence of cyanide.

The experiments with Lucifer yellow clearly demonstrate that both the number of fusion events and the number of cells involved in one dye coupling event increase as a function of time. This result favors the conjecture that SFV-induced cell-cell fusion is not a strictly synchronised process. It can be assumed that fusion proceeds in a zipper-like fashion and that a postulated cascade of correlating biochemical processes would also be asynchronous. This could explain the fact that potassium cyanide can be added even 4 minutes after triggering SFV-induced fusion in order to prevent a massive

fusion as seen in phase contrast microscopy. However, a concomitant addition of potassium cyanide with the triggering by low pH totally abolished the fusion event. Therefore, it can be concluded that potassium cyanide inhibits SFV-induced polykaryon formation at the level of membrane-membrane fusion. Potassium cyanide is an inhibitor of oxidative phosphorylation and leads to depletion of cellular ATP. For the following reasons it can be excluded that the results obtained in presence of the relative high concentration of potassium cyanide (1 mM) represent an artefact: i) polykaryon formation occurred in presence of 1 mM KCN when the drug was added 5 to 10 minutes after lowering the pH and proceeded from a microscopically invisible to a visible state (see Table 1); ii) furthermore, we have previously shown that syncytium formation of SFV infected *Aedes* cells takes place also in presence of 1 mM KCN if the infected cells are exposed to pH 6 for a short time only (4). Briefly, 16 hours pi SFV-infected *Aedes* cells were exposed for various times (1, 5 and 10 minutes) to medium of pH 6 containing 1 mM KCN. Then the medium was replaced with medium of pH 7 also containing 1 mM KCN. At exposure times of 1 and 5 minutes this treatment lead to a transient recovery of the intracellular ATP, which was sufficient for polykaryon formation to occur. In contrast, longer exposures to pH 6 in presence of KCN inhibited the fusion process. Thus, the results reported herein strongly support the finding from our laboratory that SFV-induced cell-cell fusion is an event which requires a specific expenditure of energy (4). This finding is in agreement with an earlier observation made by Okada (8). It was reported that Sendai virus-induced polynuclear cell formation of Ehrlich's ascites tumor cells was inhibited by the addition of dinitrophenol, a well known inhibitor of oxidative phosphorylation.

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