Fusion between Sendai virus envelopes and biological membranes as monitored by energy transfer methods

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Chlorophyll *a* and chlorophyll *b* have been inserted into reconstituted envelopes of Sendai virus particles. Fluorescence measurements indicated a high efficiency of energy transfer between the two chlorophyll molecules due to their close proximity in the viral envelope. Fusion of reconstituted, pigmented virus envelopes with various biological cell membranes at 37 °C resulted in a significant decrease in the yield of energy transfer. Reduction in the efficiency of energy transfer was temperature and time dependent, and was also dependent upon the ratio between the reconstituted Sendai virus envelopes (donor) and recipient cells (acceptor). No reduction in the efficiency of energy transfer was observed when non-fusogenic, reconstituted viral envelopes were incubated with cell membranes.

Membrane fusion *Sendai virus* *Energy transfer*

1. INTRODUCTION

Entry of enveloped viruses into animal cells, especially those belonging to the paramyxovirus group such as Sendai virus particles, occurs following fusion of the viral envelope with the plasma membrane of the recipient cell [1]. Reconstituted viral envelopes, which are empty phospholipid vesicles containing the Sendai virus envelope glycoproteins, are able to, similarly to intact virus particles, promote cell-cell fusion and cell lysis [2]. In addition to being an excellent tool to study structure function relationships of the viral envelope components and the molecular mechanism of virus-cell fusion, the reconstituted Sendai virus envelopes have also been used as an efficient biological carrier [3].

Evidently, to gain better understanding of the process of virus-membrane fusion as well as for the construction of highly fusogenic and efficient biological vehicle, a quantitative assay system to follow virus-cell fusion is necessary. Binding of virus particles to recipient cell membranes can be followed by the use of radiolabeled virus particles. However, many of the adsorbed virus particles fail to fuse with the membranes to which they are attached. The currently used methods for studying virus-cell fusion are semi-quantitative and indirect, based either on microscopic observation [4] or measurements of lytic processes induced by enveloped viruses [2]. Recently, energy transfer methods have been employed to follow fusion processes between phospholipid liposomes [1,5]. It has been shown that chlorophyll *a* and chlorophyll *b* molecules are highly suitable as a fluorescence couple to allow energy transfer [5]. Decrease in the efficiency of energy transfer permits quantitative estimation of membrane fusion processes.

Here, we have incorporated chlorophyll molecules into reconstituted Sendai virus envelopes. Fusion between the reconstituted, pigmented virus envelopes and cell membranes resulted in a significant decrease in the yield of energy transfer. The results obtained clearly show that energy transfer methods can be used as an assay system to follow virus-membrane fusion.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Trypsin (type III), phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were all

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purchased from Sigma. Neuraminidase (*Vibrio cholera*, 1000 units/ml) was from Behringwerke (FRG).

2.2. *Virus*

Sendai virus was propagated, harvested and suspended in 150 mM NaCl, buffered with 10 mM Tricine–NaOH (pH 7.4, solution A) as in [2].

2.3. *Cells and membrane preparation*

Human erythrocytes, type O, RH^+, recently outdated, were washed 3 times in solution A. The washed erythrocytes were hemolysed by addition of 40 vols of 5 mM phosphate buffer, pH 8.0 [6]. The final pellet of white, human erythrocyte ghosts was resuspended in solution A, to give 3 mg protein/ml. Ehrlich ascites tumor cells (ETC) were grown intraperitoneally in albino mice, as in [7].

Crude synaptic membrane preparations (P_2 fraction) were obtained from rat brains [8] and suspended in solution A to give 3 mg protein/ml.

2.4. *Preparation of reconstituted, pigmented Sendai virus envelopes*

Sendai virus particles (10 mg) were dissolved with 500 μl of 4% (w/v) Triton X-100, as in [9]. The clear supernatant which contained the viral envelope phospholipids and the two viral glycoproteins was added to an extract of spinach leaves [5]. Chlorophyll concentration in the extract was determined as in [10]. For insertion into Sendai virus envelopes, a sample of the extract containing 0.3 mg chlorophyll/ml was evaporated and the dry layers obtained were dissolved in a detergent solution of the viral glycoproteins by vigorous shaking for 5 min, after which the Triton X-100 was removed by direct addition of SM-2 Bio-beads [9]. The reconstituted, pigmented virus envelopes were centrifuged at 100000 $\times$ *g* for 30 min, and the pellet obtained was suspended in solution A to give a concentration of 2 mg viral proteins/ml. Incorporation of chlorophyll molecules into the virus envelopes was evident from experiments showing that antiviral antibodies caused precipitation of the viral glycoproteins together with the chlorophyll molecules (not shown).

Fluorescence of reconstituted, pigmented virus envelopes was measured in an MPF-44B Perkin-Elmer spectrophotometer, exactly as in [5] (excitation at 469 nm, emission at 658, 677 and 700 nm). The efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* was calculated using [5]: $(F_{677}-F_{700})/(F_{658}-F_{700})$.

Reconstituted, pigmented Sendai virus envelopes (2–5 μg viral protein) were added to a suspension containing 3×10^7 human erythrocyte ghosts, or 1×10^6 ETC, or 3 mg of the synaptic membrane preparation at room temperature (21°C), in a final volume of 1.5 ml solution A. After estimation of the fluorescence degree (zero time of incubation), the various systems were incubated at 37°C. At the end of the incubation period, the degree of the fluorescence in the systems was monitored again at room temperature (21°C). Protein was determined as in [11], using bovine serum albumin as a standard. Phospholipid was determined according to [12].

3. RESULTS AND DISCUSSION

It was recently shown that when chlorophyll *a* and chlorophyll *b* molecules were present at the appropriate surface density within the same phospholipid bilayer (liposome), energy transfer between the two chlorophyll molecules was possible [5]. Fusion between pigmented and non-pigmented liposomes resulted in a reduction in the efficiency of the energy transfer due to dilution of the chlorophyll molecules. After fusion, an increase in the emission spectra of chlorophyll *b* (618 nm) was observed [5].

Chlorophyll *a* and *b* can be inserted into the membranes of reconstituted Sendai virus envelopes, if a spinach extract is added to a detergent solution of the viral glycoproteins. Removal of the detergent resulted in the formation of resealed vesicles containing the viral envelope components (glycoproteins and phospholipids) and the chlorophyll molecules within the same membrane.

Incorporation of the pigments into the reconstituted viral envelope is evident from the spectrum analysis of the vesicles formed after removal of the detergent (fig.1). As shown, most of the light was emitted at a wavelength characteristic of chlorophyll *a*, although the membrane vesicles were excited at the absorption wavelength of chlorophyll *b*. This indicates high efficiency of energy transfer due to the close proximity of the two chlorophyll molecules. After dilution of the chlorophyll molecules by solubilization of the

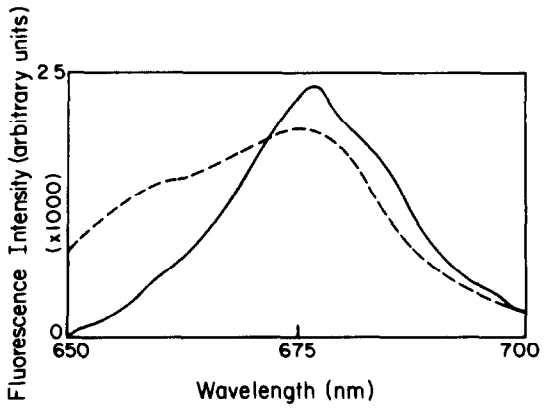

Fig.1. Emission spectra of reconstituted, pigmented Sendai virus envelopes before (——) and after (– – –) solubilization with Triton X-100.

vesicles with Triton X-100, a significant increase in the light emitted at a wavelength of chlorophyll *b* was noted (fig.1).

The results in fig.2 show that up to 4 μg chlorophylls can be added per 100 μg viral lipids

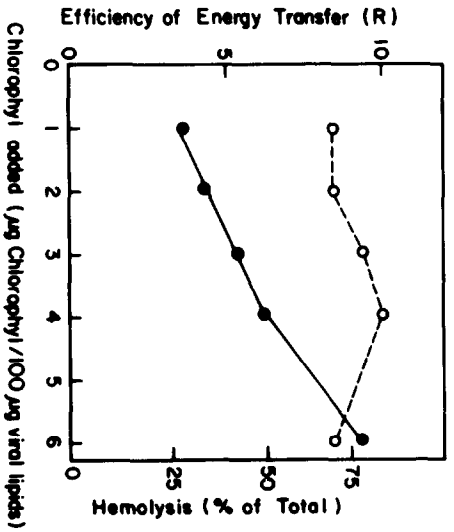

Fig.2. The effect of increasing concentrations of chlorophyll on the hemolytic activity of reconstituted, pigmented Sendai virus envelopes. Sendai virus envelopes were reconstituted in the presence of increasing concentrations of chlorophyll. Efficiency of energy transfer (——) was recorded for each reconstitution system. Hemolysis (– – –) was induced by incubating 2 μg reconstituted, pigmented envelopes with washed human erythrocytes (2.5%, v/v, in a final volume of 100 μl solution A) for 10 min at 37°C. Hemolysis was estimated at 540 nm, as in [2].

(4%, w/w, of the total viral lipids) without impairing the viral hemolytic activity. Addition of higher concentrations of chlorophyll caused partial inhibition and, in the presence of 10% chlorophyll/total viral lipids, complete inhibition of the viral hemolytic activity was observed (not shown). It should be emphasized that virus-induced hemolysis reflects a process of virus-cell fusion [4].

The results in table 1 show that incubation of reconstituted, pigmented Sendai virus envelopes with human erythrocyte ghosts (HEG), ETC or with brain synaptic membrane preparations at 37°C, resulted in a significant reduction in the efficiency of energy transfer. This probably reflects fusion between the viral envelopes and the membranes of HEG, ETC or those obtained from rat brains, processes which will lead to dilution of the chlorophyll molecules.

No reduction in the efficiency of energy transfer was observed when either trypsinized or PMSF-treated, reconstituted virus envelopes were incubated at 37°C with the various membrane preparations (table 1). Both treatments are known to inactivate specifically the virus fusogenic activity, without impairing its binding ability [13]. Reduction in energy transfer also did not occur when DTT-reduced reconstituted envelopes were incubated with HEG or ETC (table 1). The same results were obtained when the reconstituted, pigmented vesicles were incubated with desialized HEG (table 1). DTT-reduced Sendai virus particles are unable to attach to cells and therefore cannot fuse with their membranes, due to inactivation of the viral binding protein [14]. It should be noted that the reduction in the efficiency of energy transfer observed after fusion of the virus envelopes with either HEG or ETC was close to that observed after solubilization of the reconstituted, pigmented envelopes with Triton X-100 (see legend to table 1). This indicates a high degree of fusion between the virus envelope and the membranes of HEG or ETC, as previously inferred from experiments using electron microscopic techniques [4,7].

Reduction in the efficiency of energy transfer was a temperature-dependent process, reaching a maximum at 34–37°C (fig.3). Very little, if any, reduction in the energy transfer was observed when the reconstituted envelopes were incubated with HEG or ETC at temperatures below 10°C (fig.3).

Table 1

Fusion between reconstituted, pigmented Sendai virus envelopes and biological membranes, as monitored by reduction in efficiency of energy transfer

System	Incubation at 37°C with					
	HEG		ETC		Brain synaptic membrane preparations	
Incubation time (min):	0	15	0	15	0	15
Reconstituted Sendai virus envelopes	5.8	2.8	5.1	2.6	5.0	3.2
Trypsinized reconstituted envelopes	5.4	5.4	6.2	5.8	N.D.	N.D.
DTT-reconstituted virus envelopes	5.0	5.1	5.2	5.3	N.D.	N.D.
PMSF-reconstituted virus envelopes	5.2	5.5	5.2	5.3	5.6	5.1
Reconstituted Sendai virus envelopes	4.9[a]	5.0[a]	N.D.	N.D.	N.D.	N.D.

[a] Reconstituted, pigmented Sendai virus envelopes were incubated with desialized HEG. Membrane sialic acid residues were removed from HEG by treatment with neuraminidase as in [15]

N.D., not done. Reconstituted virus envelopes (2.5 μg) were incubated at 37°C with HEG, ETC or brain synaptic membrane preparations, as described in section 2. Reconstituted Sendai virus envelopes were treated with trypsin, PMSF and DTT as in [6,15]. The extent of efficiency in energy transfer obtained after solubilization of the reconstituted envelopes with Triton X-100 (0.1%) was 2.2

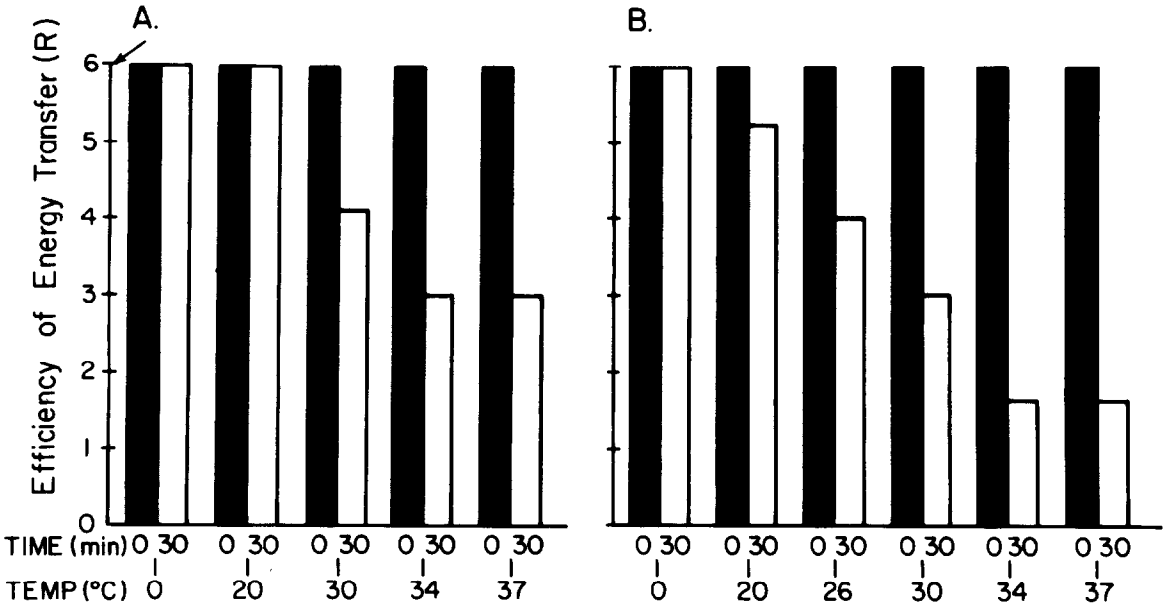

Fig.3. Reduction in the efficiency of energy transfer during incubation of reconstituted, pigmented Sendai virus envelopes with HEG and ETC: Effect of temperature. Reconstituted, pigmented Sendai virus envelopes (3 μg) were incubated with (A) HEG (3×10^7) or (B) ETC (10^6) for 30 min at the indicated temperature. Fluorescence intensities were recorded at the beginning (zero time) and at the end of the incubation period. All fluorescence measurements were performed at room temperature, except for the sample incubated at 0°C. For the latter, the fluorescence intensities were recorded at 4°C. No changes in the efficiency of energy transfer were observed between 0 and 37°C with reconstituted, pigmented Sendai virus envelopes incubated in the absence of cells. The value of the efficiency in energy transfer of reconstituted, pigmented envelopes is indicated by the arrow.

Kinetic analyses showed that most of the reduction in the efficiency of energy transfer occurred within the first 4 min of incubation at 37°C (fig.4). It seems that under the conditions used, the process of virus-membrane fusion, as reflected by the reduction in the efficiency of energy transfer, was completed within 13–15 min of incubation at 37°C (fig.4).

The results in fig.5 show that the reduction in the efficiency of energy transfer was dependent upon the ratio between the reconstituted Sendai virus envelopes (donor) and the recipient cells (acceptor). When 1.5–4.5 μg reconstituted envelopes were incubated with 10^7 HEG, about 55% reduction in the efficiency of energy transfer was observed after 15 min incubation at 37°C (fig.5). However, when 6 μg reconstituted envelopes were incubated with the same number of HEG, the reduction in the efficiency of energy transfer was less pronounced, reaching about 40% (fig.5). At higher virus concentrations (12–24 μg/10^7 HEG), reduction in the efficiency of energy transfer was negligible (fig.5). Longer periods of incubation did not significantly alter this result (not shown). These results probably indicate that at a high ratio of reconstituted envelopes (or intact virus particles) per recipient cell, a large proportion of the reconstituted envelopes present in the suspension are unable to fuse with the erythrocyte membranes. Indeed, previous experiments in our laboratory have shown that the number of virus particles which are able to fuse with each human

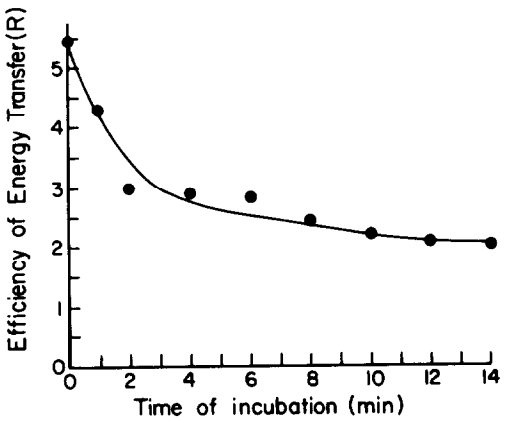

Fig.4. Kinetic studies of the reduction in the efficiency of energy transfer observed during fusion of reconstituted, pigmented Sendai virus envelopes and HEG.

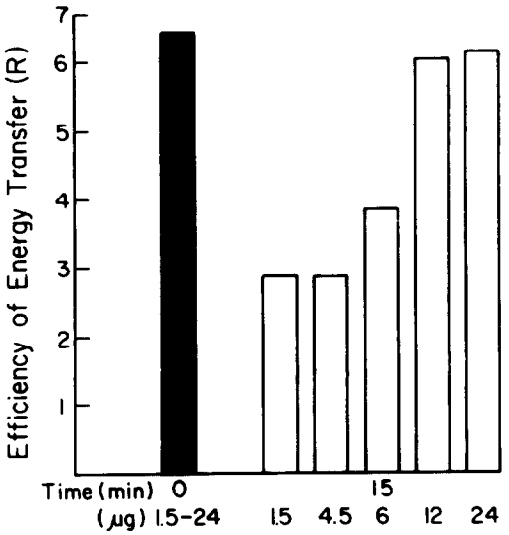

Fig.5. Changes in the efficiency of energy transfer during reconstituted, pigmented Sendai virus envelopes with HEG: Effect of virus concentration. Increasing concentrations of reconstituted, pigmented Sendai virus envelopes were incubated with 3 × 10^7 HEG. Fluorescence intensities were recorded before (zero time) and at the end (15 min) of the incubation period.

red blood cells (or other cells in culture) is limited, reaching about 700–1000 virus particles/human erythrocyte [6].

The results of this work show, for the first time, the use of an energy transfer method to follow the process of virus-membrane or virus-cell fusion. This method will potentially allow a better quantitation of the process of virus-membrane fusion and permit studies of fusion processes between virus envelopes and pure membrane preparations. Currently, attempts are being made in our laboratory to incorporate chlorophyll molecules or other fluorescent probes into envelopes of intact Sendai virus particles, thus allowing fusion studies between intact virus particles and cell membranes.

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