Biological Activities of Sonically Treated Sendai Virus

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SUMMARY

Sendai virus induced fusion of Ehrlich ascites tumour cells but this capacity was decreased by sonic treatment and the product then interfered with virus-induced fusion. Sucrose density gradient fractionation of the sonic product showed that the capacity for fusion resided in intact particles and the interfering effect in fragments of the virus envelope. Such fractionation also showed that haemolytic activity was restricted to intact particles or to large envelope fragments, while haemagglutination is found with large or small fragments. However, envelope fragments, with haemolytic activity, induced fusion in cell monolayers with little or no inhibitory effect. Envelope fragments complexed with antibody lose their capacity to inhibit fusion and show no capacity for fusion of Ehrlich ascites tumour cells.

These findings, and studies by electron microscopy on interactions of envelope fragments to cells, support the hypothesis that the fusion of suspended cells depends on the strength of contact between cells induced by virus components with haemolytic activity.

INTRODUCTION

Haemagglutinating, haemolytic and cell fusion activities of Sendai virus (HVJ, Haemagglutinating Virus of Japan) are associated with the envelope of the virion. These activities were studied after disruption of virus particles by sonic treatment. Sonically disrupted Sendai virus interfered with virus-induced fusion and the cell fusion activity for cells in suspension differed from that for cell monolayers.

METHODS

Virus. Sendai virus (HVJ), z strain, was used throughout. The virus was inoculated into the allantoic cavities of 10-day-old eggs (white Leghorn) and the infected allantoic fluids were harvested after incubation for 3 days. Sendai virus particles were partially purified from the infected fluids by differential centrifugation (2500 rev./min. for 20 min. and 20,000 rev./min. for 30 min.) and suspension in SSC (0.15M-NaCl + 0.015M-sodium citrate). The partially purified particles were resuspended in PBS (phosphate buffered saline, pH 7.2) for sonic treatment or for cell fusion, using a similar procedure of differential centrifugation.

Cells. Ehrlich ascites tumour cells (hypertetraploid) of adult ddO mice were harvested, washed three times with Eagle's medium and suspended (10% v/v) in the same medium with 2% calf serum.
KB, FL, Vero and BHK21 cells were grown in Eagle's medium with 10% calf serum. Monolayers of these cells were washed with PBS, detached from the glass wall by treatment with 0.05% trypsin and 0.2% Versene in PBS and centrifuged at 800 rev./min. for 5 min. The sedimented cells were suspended in Eagle's medium with 2% calf serum, filtered through several layers of gauze and centrifuged again. After resuspension in the same medium to give a 10% (v/v) suspension the cells were used for experiments on fusion in suspensions. More than 90% of the cells in suspension were single.

Monolayers of the same cells were also detached as described above and inoculated on cover slips in Leighton tubes. One or two days later the resulting monolayers were used in experiments on fusion.

Assays of infectivity, haemagglutinating activity (HA) and haemolytic and cell fusion activities. The assay of infectivity, HA and haemolytic activity were described in the previous report (Hosaka, Kitano & Ikeguchi, 1966). In experiments on fusion with cells in suspension, 0.8 ml. of Ehrlich ascites tumour cells or 0.4 ml. of other cells were mixed with an equal volume of virus sample in an ice bath for 15 min. and then incubated at 37° for 45 min. The cells were then counted and the fusion index (Okada & Tadokoro, 1962) determined. Further incubation did not increase the extent of cell fusion.

Cell monolayers were washed and inoculated with 1.5 ml. of virus sample diluted twofold with Eagle's medium containing 1% calf serum. After incubation at 37° for 3 hr the cells were stained with Giemsa's solution and the number of cells and nuclei counted. The fusion index for cell monolayers was expressed as:

$$\frac{\text{number of nuclei}}{\text{number of cells}}$$

for test sample

$$-\frac{\text{number of nuclei}}{\text{number of cells}}$$

for control sample.

Unless otherwise stated, the cell fusion activity of Sendai virus is expressed in relation to Ehrlich ascites tumour cells.

Estimation of interference for cell fusion. After sonic treatment of Sendai virus 0.4 ml. of suspension in PBS was mixed with an equal volume of untreated virus (10,000 or 20,000 HAU/ml.), the mixture was added to 0.8 ml. of Ehrlich ascites tumour cells in the cold and then incubated at 37° as in the assay for cell fusion. Interference with cell fusion by the test sample was expressed by the fusion index of this system as compared with that for Sendai virus alone.

Determination of protein content. Protein content was measured by the method of Lowry et al. (1951).

Fractionation by sucrose density gradient centrifugation. Gradients were formed from 5 ml. each of 15, 18, 22, 26 and 30% sucrose (w/v) in SSC. Samples were layered on top of the gradients and the tubes were centrifuged in the cold at 17,000 rev./min. for 70 min. in an SW25 rotor. Drop fractions were collected from the bottom of the tubes.

Sonic treatment of Sendai virus. Samples in PBS were treated in the cold by an ultrasonic oscillator (Kaijo Denki, type 4210, 150W, 20 kyecc./sec.).

Preparation of anti-Sendai rabbit serum. The method was described previously (Hosaka, 1968).

Electron microscopy. Particles with envelopes and envelope fragments were adsorbed to red cells (Ishida, Amano & Kitawa, 1962) and then examined on a Hitachi (type HUII B) electron microscope after negative staining by 1% phosphotungstic acid (pH 7.2).

Thin sections of Ehrlich ascites tumour cells with virus components were made and examined as in the Sendai virus–Ehrlich ascites cell system (Hosaka & Koshi, 1968).
RESULTS

Interference with cell fusion by sonically treated Sendai virus

The activity of Sendai virus in inducing fusion of Ehrlich ascites tumour cells could be inhibited by sonically treated virus (Okada & Tadokoro, 1962). On incubation of Ehrlich ascites tumour cells with intact virus at 37°, subsequent addition of treated virus even within 30 sec., had no significant effect on cell fusion. However, the addition of a relatively low concentration of treated virus before untreated virus interfered with cell fusion. Since the pH of the medium fell until the untreated virus was added, a condition unfavourable for cell fusion, we adopted the procedure of simultaneous addition of treated and untreated virus to cells in the cold, followed by incubation at 37°. In this interference system small aggregates of cells were formed during the absorption period in the presence of high concentrations of treated virus and large aggregates of cells were formed in its absence.

As anticipated, treated virus with haemolytic and haemagglutinating activity did not interfere with virus-induced haemolysis or haemagglutination.

![Graph showing alteration of virus activities during sonic treatments](image)

Alteration of virus activities during sonic treatments

Alterations of infectivity, HA, haemolytic and cell fusion activities, virus opalescence at 550 nm. and of development of the interfering effect were measured at two levels of active Sendai virus (Fig. 1 a, b). Infectivity and opalescence decreased to about half after sonic treatment for 20 min., indicating considerable disruption of virions. At the same time, the HA doubled and the haemolytic activity increased even more, irrespective of virus concen-
The cell fusion activity decreased relatively rapidly, although long treated virus still interfered with cell fusion. The small amount of interfering activity due to untreated virus is discussed later.

**Sucrose density gradient fractionation or sonically treated Sendai virus**

After sonic treatment for 20 min. Sendai virus was fractionated by sucrose density gradient centrifugation and the distribution of HA, haemolytic and cell fusion activity, infectivity and protein content determined (Fig. 2a, b). For the untreated virus the peaks of all the activities almost coincided, although the haemolytic and fusion activities were elevated near the base of the tube. This is probably because these fractions contain large particles with high haemolytic and fusion activity in relation to HA (Hosaka et al. 1966). This distribution pattern for untreated virus suggests that all the activities tested are associated with normal virus particles. The distribution of protein also followed that for HA.

For sonically treated Sendai virus the peaks of the activities were relatively widely separated. The HA showed two peaks, the more dense of which corresponded to the peak of infectivity. Haemolytic activity showed a broad peak at a density above the lighter HA peak. The peak of fusion activity corresponded approximately with that of infectivity. These results indicate that the cell-fusion activity of Sendai virus for Ehrlich ascites tumour cells is associated primarily with intact virus particles: haemolytic activity and HA are associated with disrupted particles of lighter density.

Most protein was found in the top fractions. The total protein of control fractions (Fig. 2) is less than that of treated fractions because some large virions were sedimented and not measured in the control but were disrupted and became less dense during sonic treatment.
Following longer sonic treatment the HA, infectivity and fusion activity of the base of the tube were reduced. The HA near the top of the tube was increased, but the nature of the distribution of activities was unchanged. On occasions some fractions near infective fractions induced cell lysis, although the reason for this is uncertain.

**Morphology of components showing fusion, haemolytic and haemagglutinating activity**

Red cell-adsorbed particles in fractions of sonically treated Sendai virus (Fig. 2b) were examined by electron microscopy. The fractions near the base of the tube showed fusion activity and consisted of intact particles while the upper fractions consisted of membrane particles without nucleocapsids: there were, however, membrane particles with disrupted nucleocapsids in a few upper fractions near the infective fractions. Particles in the infective fractions were 140 to 190 nm. in diameter, particles in the peak of haemolytic activity were 100 to 160 nm. in diameter and those in the upper peak of HA, 80 to 110 nm. There was also a number of linear particles of various lengths (25 to 80 nm.) with ‘spikes’ on one or both sides in the fractions of the upper peak of HA. The top fractions at the peak of protein content contained many ring structures of fragments of disrupted nucleocapsids, small fragments of envelope and other unidentified fragments.

This morphology is consistent with the distribution of activities (Fig. 2b).

![Dose response curves of interference with cell fusion (Ehrlich ascites tumour cells) by four pooled fractions derived from (see text) sonically treated (30 min.) Sendai virus. The cell fusion index for challenged intact Sendai virus alone is taken as 100%.

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**Interference with cell fusion by fractions of sonically treated Sendai virus**

The fractions of treated virus (Fig. 2b) were pooled in groups to give four new fractions: fraction 1 across the peak of infectivity, fraction 2 across the intermediate tubes between the peaks of infectivity and haemolytic activity, fraction 3 across the haemolytic peak and fraction 4 across the lower density HA peak. These four fractions were diluted with distilled water and centrifuged at 30,000 rev./min. for 1 hr. The pellets were suspended in a small volume of PBS and assayed for interference with cell fusion.

Fig. 3 shows the dose response curves for interference with virus-induced fusion. The interfering activity for fraction 1 (intact particles) was weak, but that for fractions 2, 3 and 4...
(disrupted particles) was strong. The interfering activity adjusted to the same HA level increased in the order 4, 3, 2.

**Effect of antiserum on cell fusion by sonically treated Sendai virus**

Okada, Yamada & Tadokoro (1964) reported that the Sendai virus + antibody complex formed by an appropriate amount of antiserum enhanced the fusion of Ehrlich ascites tumour cells. A study was therefore made of the behaviour of envelope fragment + antibody complexes. The four pool-fractions of the preceding section were incubated at room temperature with twofold serial dilutions of anti-Sendai rabbit serum. After 6 hr with gentle shaking the fusion activities of the mixtures were tested. The antibody-treated fractions 3 and 4 did not induce cell fusion; antibody-treated fraction 2 induced some fusion and the antibody-treated fraction 1 enhanced fusion. The complexes of fractions 2, 3 and 4 showed reduced interfering activity for fusion.

**Fusion of monolayer cells by sonically treated Sendai virus**

Table 1 shows the difference in the effect of sonic treatment on the fusion activity of Sendai virus for monolayers and suspensions of four established cell lines. The effect of sonic treatment was less when tested on cell monolayers.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Untreated Sendai virus</th>
<th>Sonically treated Sendai virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suspension</td>
<td>Monolayer</td>
</tr>
<tr>
<td>KB</td>
<td>9*</td>
<td>&gt;20*</td>
</tr>
<tr>
<td>FL</td>
<td>13</td>
<td>&gt;20</td>
</tr>
<tr>
<td>BHK 21</td>
<td>15</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Vero</td>
<td>10</td>
<td>&gt;20</td>
</tr>
</tbody>
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Sendai virions (30,000 HAU/ml.) were sonically treated for 30 min., mixed with an equal volume of Eagle's medium containing 2% calf serum and then tested for fusion of the four kinds of cell. Untreated Sendai virus was diluted and tested similarly.

* Fusion index.

The influence of sonic treatment of Sendai virus on fusion activity for suspensions and monolayers of KB cells, using Ehrlich ascites tumour cells as control, is shown in Fig. 4. The activity for KB cell suspension decreased like that for Ehrlich tumour cells; when tested on monolayers of KB cells the decrease was less than that for the control. However, the rate of decrease of fusion activity for monolayers of KB cells cannot be quantified readily, owing to the tendency to form giant cells over the whole monolayer. A similar effect was observed for the other cell lines.

Sonically treated Sendai virus interfered with virus-induced fusion in suspensions of these established cell lines; in monolayers the effect was uncertain.

When the distribution of fusion activity for these monolayer cells was examined by sucrose gradient centrifugation, as in the experiment shown in Fig. 2, the fusion activity was widely distributed. Fig. 5 for KB monolayers is typical. The upper limit of fusion activity was close to that for the distribution of haemolytic activity and different from that for fusion activity in suspensions of KB cells which corresponded to that for infective fractions. If centrifuged on sucrose, the upper haemolytic fractions similarly induced fusion in KB
Cell fusion by fragments of Sendai virus

Fig. 4. Comparison of alterations of fusion activities for KB suspended (KB) and monolayer cells (KB') of Sendai virus during sonic treatment. Ehrlich ascites tumour cells (ETC) were used as control. The initial HA of the Sendai virus sample was 16,000 HAU/ml. O—O, KB'; O—O, KB; ●—●, ETC.

Fig. 5. Distributions of fusion activities of sonically treated Sendai virus by sucrose gradient centrifugation in the cold (15 to 30%, w/v in SSC; 17,000 rev./min. for 70 min.) for KB cells in monolayers (△—△) and suspensions (▲—▲). The distributions of haemolytic activity (○—○) and HA (●—●) are also shown. Haemolytic activity was determined with 0.5 ml. of 1/20 dilutions of fractions.
Fig. 6. Adsorption of envelope fragments to Ehrlich ascites tumour cells in the cold. Envelope fragments (40,000 HAU/ml.) were prepared by differential centrifugation (12,000 rev./min. for 20 min. and 40,000 rev./min. for 60 min.) with suspension in Eagle's medium. Fragments were mixed with Ehrlich ascites tumour cells (1.2 x 10⁷ cells/ml.) in Eagle's medium with 2% calf serum in the cold. Linear and closed envelope fragments are indicated by I and c, respectively.

Fig. 7. Adsorption of intact virus particles to Ehrlich ascites tumour cells in the cold. Partially purified virus (20,000 HAU/ml.) was mixed with the cells as for the envelope fragments. Sendai virus particles are indicated by v.
Cell fusion by fragments of Sendai virus

monolayers; this eliminated the possibility that the fusion activity in these fractions was due to contamination by intact virus.

Electron microscopy of interaction of Ehrlich ascites tumour cells with envelope fragments

It was of interest to consider why envelope fragments with haemolytic activity induced little fusion of Ehrlich ascites tumour cells but significant fusion in cell monolayers. Since cell contacts are a prerequisite for cell fusion, then cells in suspension require a virus activity to establish contact between cells before fusion can occur: this is not necessary in cell monolayers where cells are already in contact. In the latter case, if a viral component absorbs to cells, then its only necessary activity will be to modify the cell membranes in order to form a cytoplasmic bridge. Fusion activity for monolayer cells may be the same as haemolytic activity because both are distributed similarly following sucrose gradient centrifugation (Fig. 5). Thus, it may be that only virus components with haemolytic activity and cell-contacting ability above a certain level, i.e. intact virus particles, can induce fusion of cells in suspension.

In electron micrographs (Fig. 6) of envelope fragments absorbed to Ehrlich ascites tumour cells in the cold, the linear and closed fragments have a small area of contact with cell membranes, in contrast to the contact of intact virus particles (Fig. 7). After incubation at 37°, the agglutinated cells rapidly dissociated and many fragments eluted, probably through failure to establish contact between cells. These findings support the hypothesis that only intact virus particles induce fusion of suspended cells. No envelope fragments were engulfed into the cytoplasm during incubation at 37° for 1 hr.

In interference with cell fusion, a similar finding to that of Fig. 6 was observed, although intact virus particles also adsorbed to cells. In this case fragments did not appear to inhibit adsorption of particles. Thus, in interference with fusion, the contact of two cells is probably inhibited because membrane sites on adjacent cells near already absorbed virions are occupied by envelope fragments and the membrane sites cannot make physical contact. It follows on this consideration that interference with fusion of monolayer cells cannot occur since cell contacts are already established.

Cell fusion interfering activity of particles disrupted by Emasol–ether treatment

Sendai virus was disintegrated by Emasol + ether treatment (Hosaka, Hosokawa & Fukai, 1959) and disrupted particles separated by differential centrifugation (2500 rev./min. for 20 min. and 40,000 rev./min. for 30 min.). This disrupted preparation had a high HA but neither haemolytic nor fusion activity for suspended or monolayer cells. The preparation interfered with virus-induced fusion in cell suspension but not in cell monolayers. It did not interfere with haemolysis by virus. Similarly, the lower density fraction of HA from sonically treated Sendai virus with little haemolytic activity interfered with fusion of suspended cells but not with haemolysis by virus.

DISCUSSION

Models of different types of active particles seen in fractions of sonically disrupted Sendai virus following separation by sucrose gradient centrifugation are shown in Fig. 8. Particles to the left occur at the bottom of the tube. The distribution of activities, as found in the fractions, is indicated by arrows.

Since sonically treated Sendai virus consists of particles heterogeneous in morphology and activity, any expression of activity in the treated virus must be considered as a total of the
activities of different particles and fragments. For example, little fusion activity in suspended cell systems reflects the addition of fusion activity due to intact particles and its inhibition by disrupted particles. Such fusion activity was enhanced when these particles were aggregated with antiserum (Okada & Murayama, 1968). This is due, at least in part, to enhancement of fusion activity of intact particles and reduction of inhibitory activity of disrupted particles, as shown in present experiments.

The initial small haemagglutinins of measles virus show haemolytic activity and can induce fusion of Lu 106 cells in monolayers (Norrby et al. 1964); an observation which seems to be consistent with present findings for envelope fragments with haemolytic activity from Sendai virus.

Small envelope fragments have HA but little haemolytic activity. Envelope fragments with and without gross haemolytic activity differ only in diameter and by less than 50 nm. The two types of fragment have a similar density of surface spikes. In small haemagglutinins, a co-operation of neighbouring spikes may be difficult owing to the distance of the tops of radially projecting spikes, which may make haemolytic factors inactive. Alternatively, the haemolytic factor (Neurath, 1965) may have been removed by the sonic treatment.

It is necessary to consider why fusion of suspended cells was inhibited by envelope fragments lacking haemolytic activity while haemolysis was scarcely influenced. Haemolysis occurs following interaction of virus components with the single red cell, but fusion follows the interaction of virus components with two cells in contact. If envelope fragments inhibit cell contact but not absorption of virus components they would not inhibit haemolysis.

High concentrations of untreated (Fig. 1) or undisrupted (Fig. 3) virus particles have slight inhibitory effect on virus-induced fusion. Okada & Tadokoro (1962) reported that con-
centrations of Sendai virus above a certain level reduced cell fusion. When many particles absorb between adjacent cells, stable contacts between the cell membranes may be prevented and lead to interference of fusion.

The higher haemolytic and fusion activities of large Sendai virus particles (Hosaka et al. 1966) must be explained by distinct mechanisms: haemolytic activity is due to the formation of a large area of contact between virus and red cell membrane, but fusion activity is due to the induction of a more stable contact between two cells by a large area of contact with virus.

Many suggestions that cell fusion and haemolysis by viruses have a common mechanism have been reported (Bukrinskaya & Zhadanov, 1961; Cascardo & Karzon, 1965; Henle, Deinhardt & Girardi, 1954; Ho Yun-de, 1962; Hosaka, 1962; Kohn, 1965; Norrby et al. 1964; Okada & Murayama, 1966; Russell & Morgan, 1959). The present results support this hypothesis for Sendai virus only for early cell fusion. Sato (personal communication) showed that Sendai virus without haemolytic activity (grown in GM cells, a continuous line of Green monkey kidney cells) induced late fusion of cells but not early fusion. This effect was associated with virus multiplication.

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