Trypsin Action on the Growth of Sendai Virus in Tissue Culture Cells

II. Restoration of the Hemolytic Activity of L Cell-Borne Sendai Virus by Trypsin

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Sendai virus grown in L cells (L Sendai) caused little hemolysis, whereas the one grown in fertile eggs (egg Sendai) induced distinct hemolysis. Enzymatic treatment with trypsin at low concentrations markedly enhanced the hemolytic activity of L Sendai but not that of egg Sendai. Both sonic treatment and freezing and thawing greatly enhanced the hemolytic activity of egg Sendai, but they gave little enhancing effect on that of L Sendai which could, however, be greatly increased by successive treatment with trypsin. Dose response and kinetic experiments on the trypsin effect have suggested that a similarity exists in the inhibitory mechanism of infectivity for L cells and hemolytic activity of L Sendai. Treatment of L cells with trypsin at later stages of infection released a highly hemolytic L Sendai from those cells. The present study, by reference to the density centrifugation studies in a previous report (4), has shown that a variation in infectivity for L cells and in the hemolytic activity of L Sendai is a type of host-controlled modification distinguishable from the density variation.

In previous papers (3, 4, 8, 9), the differences between egg-borne Sendai virus (egg Sendai) and L cell-borne Sendai virus (L Sendai) in their biological and physical properties were described, and these phenomena were attributed to host-controlled modifications: L Sendai is apparently lacking in infectivity for L cells and hemolytic activity and has a higher density. I have found that trypsin treatment of L Sendai at a low concentration increased the infectivity of L Sendai for L cells 1,000-fold without changing the morphological integrity and density (4).

To explain the inhibitory mechanism involved in the expression of the infectivity of L Sendai for L cells, a "masking substance" was operationally proposed which may be taken up by L Sendai on its surface from the host cell in the course of its maturation. The trypsin treatment of L Sendai may remove such a substance selectively from the virion and render the virus infectious for L cells.

The present paper describes how hemolytic activity of L Sendai is markedly enhanced by treatment with trypsin at low concentrations. The condition which induces the maximal enhancing effect is also determined, and acceptability of the above masking substance hypothesis in relation to the restoration of the hemolytic activity of L

Sendai is discussed. Concerning the inhibitory mechanism of L Sendai, the similarity between the biological expression of infectivity and hemolysis is disclosed. A more direct approach is attempted to support the idea of masking substance in which hemolytic L Sendai is released by treatment of the infected L cells with trypsin. A comparison of the enhancement of the hemolysis of egg and L Sendai by trypsin and sonic treatment is made, and a description of the functional structure of the hemolytic unit of Sendai virus is attempted.

MATERIALS AND METHODS

Virus. The Fushimi strain of Sendai virus was used. Preparation and partial purification of egg Sendai and L Sendai have been described (4). All the viruses tested were suspended in phosphate-buffered saline (PBS), pH 7.2.

Treatment of Sendai virus with trypsin. To 0.3 ml of virus suspension in a test tube (10 by 100 mm) was added 0.1 ml of crystalline trypsin in PBS in an ice water bath. Enzymatic treatment was initiated in a water bath at 36°C. To induce an immediate stop of the enzyme action, the virus-trypsin mixtures were chilled in ice, to which then was added 0.1 ml of soybean trypsin inhibitor at a concentration more than 10 times that of the trypsin used in the respective experiments.

Sonic treatment of Sendai virus. Samples in PBS

were treated in ice with an ultrasonic oscillator (Tominaga, model UR-150P, 133 w, 20 kc/sec).

Freezing and thawing of Sendai virus. To perform this particular experiment, viruses were freshly prepared as described (4) and kept cold, but not frozen. Samples to be tested were divided among the test tubes. Freezing and thawing was accomplished by immersing the tubes containing virus suspensions alternately into a mixture of dry ice and ethanol and into a water bath at 36 C.

Treatment of infected L cells with trypsin. Petri dish cultures of L cells were infected with egg Sendai at a multiplicity of 10. Adsorption, washing of the residual virus, and feeding with maintenance solution (MS) were the same as described (4). At indicated times after infection, the monolayers were washed with prewarmed PBS and immersed in 0.005% trypsin (Nutritional Biochemicals Corp.), 1:300, for 1 min at 36 C. The trypsin was aspirated and MS containing soybean trypsin inhibitor was immediately replaced.

Measurement of hemolysis. To 0.5 ml of virus suspensions in the test tube was added 2 ml of 2% guinea pig red blood cell suspensions in PBS. Incubation was in a water bath at 36 C for 40 min with occasional manual shaking. At the end of the incubation period, the tubes were centrifuged, and the supernatant fluids were measured for hemolysis according to their optical density at 575 nm.

Chemicals. Crystalline trypsin and crystalline soybean inhibitor were the same as described previously (4).

RESULTS

Restoration of the hemolytic activity of L Sendai by trypsin. As reported previously, the hemolytic activity and infectivity for L cells of L Sendai have largely been depressed, this phenomenon being considered a host-controlled modification (3, 8, 9). The infectivity of L Sendai for L cells was recovered remarkably by treatment with trypsin at low concentrations (4). It was of interest therefore to investigate whether the same treatment of L Sendai induces restoration of hemolytic activity.

L Sendai was treated with trypsin at a final concentration of 0.0001% at 36 C. After various periods of time, soybean trypsin inhibitor was added to the reaction mixture, and then the hemolytic activity was measured (Fig. 1).

Only a very low level of hemolysis was caused by L Sendai which had not been treated with trypsin (control). Restoration of the hemolytic activity of L Sendai was induced immediately after the addition of trypsin with a rapid rate of increase. Maximum levels were reached between 6 and 10 min, and the prolonged incubation resulted in a gradual decrease. A complete and immediate abolishment of the trypsin effect by soybean inhibitor was evidently shown because the incorporation of the inhibitor into the reaction mixture at the time of addition of trypsin (zero time) did not result in any restoration of the hemolytic activity of L Sendai.

FIG. 1. Kinetics of restoration of the hemolytic activity of L Sendai after exposure to trypsin. The test tubes containing 0.3 ml of L Sendai at 512 hemagglutinin units per ml were treated with trypsin at a final concentration of 0.0001% at 36 C. Soybean trypsin inhibitor was added to the reaction mixture at the times indicated. Hemolytic activity was measured as described in Materials and Methods. In a control experiment, the trypsin treatment was omitted (shown by arrow).

Comparative study of trypsin effect on the hemolysis of egg and L Sendai. Since little enhancing effect of trypsin on the infectivity of egg Sendai for L cells was demonstrable (4), an experiment was conducted to see the effect of trypsin on the hemolytic activity of egg Sendai. In this experiment, a comparative study of trypsin effect on the hemolysis of egg and L Sendai was made. To ensure the quantitation of hemolysis, a series of twofold dilutions of both egg and L Sendai were treated with various concentrations of trypsin at 36 C for 6 min. The results are shown in Fig. 2.

There was a linear relationship between the levels of hemolysis and virus titer in the optical density range of 0.1 to 2 under the experimental conditions, irrespective of the kind of virus used and trypsin concentration. This relationship was distorted when hemolysis was over an optical density of 2, probably because of a relative shortage of the red blood cell substrate. Distinct hemolysis was caused by egg Sendai without trypsin treatment with all of the virus dilutions used, and the minimal virus dose to require the induction of significant hemolysis was about 2 to 4 hemagglutinin units (HA) per ml under the given conditions (*not shown*). On the contrary, the hemolysis caused by L Sendai without trypsin treatment was only significant when more than ca. 100 HA per ml was used. A marked increase in the hemolytic activity of L Sendai was obtained by treatment with trypsin at any of the concentrations used, and again the linear relationship between the level of hemolysis and the virus dose was observed. However, the level of hemolysis of L Sendai, even after the maximum induction by trypsin, could not reach that of egg Sendai. To make it easy to compare the effect of trypsin on the hemolytic activity

FIG. 2. Effect of trypsin on the hemolytic activity of egg and L Sendai. Serial twofold dilutions of egg Sendai (●) and L Sendai (○) were treated with various concentrations of trypsin for 6 min at 36 C. At the end of the incubation period, soybean trypsin inhibitor was added to the reaction mixture to stop the enzymatic action. Hemolytic activity was measured as described in Materials and Methods. The final trypsin concentrations were (a) 0.0, (b) 0.000025, (c) 0.0001, (d) 0.0004, (e) 0.0016, and (f) 0.0064%.

of L Sendai with its effect on egg Sendai, Fig. 3 was derived from Fig. 2, where the grade of hemolysis caused by both egg and L Sendai at 188 HA per ml was plotted on the function of trypsin concentration. The hemolytic activity of L Sendai was increased by treatment with trypsin at very low concentrations, reached maximum activity at 0.0004%, and finally decreased as the concentration increased. Under the given conditions, none of the enhancing effect of trypsin on the hemolytic activity of egg Sendai was obtained, but rather the activity was decreasing as the concentration of trypsin increased.

Effect of sonic treatment on the hemolytic activity of egg and L Sendai. The enhancing effect of sonic treatment on the hemolytic activity of Sendai virus grown in fertile eggs has been reported (5, 11). A similar effect has also been shown with the closely related Newcastle disease virus (2). A study was therefore made to see whether sonic treatment enhances the hemolytic activity of L Sendai as equally as that of egg Sendai.

Egg and L Sendai were sonically treated for the various periods of time noted. A sample from each of the respective viruses was taken out and divided into two portions, one of which was subjected to further treatment with trypsin at a concentration of 0.0004% for 6 min at 36 C. The hemolytic activity was measured as described. The obtained results are illustrated in Fig. 4.

It is clear that the hemolysis of egg Sendai was enhanced immediately after exposure to sonic treatment. The hemolytic activity increased up to 3 min, at which time the maximum hemolysis was obtained, and decreased thereafter. A 20-min exposure completely abolished the hemolysis. A quite different pattern was obtained with L Sendai. Before sonic treatment, the starting material showed a little hemolysis. After short exposure (30 sec), a slight, but significant, increase of hemolysis was obtained. However, further treatment showed a decreasing effect. It should be emphasized here that this pattern was highly reproducible, which was confirmed by repeated experiments using a higher dose of L Sendai. There was little effect of trypsin on the hemolysis of egg Sendai after sonic treatment, which yielded a pattern similar to that obtained before trypsin treatment. The same treatment of L Sendai with trypsin after sonic treatment resulted in a marked enhancement of the hemolysis. It is of interest that the pattern thus obtained was very similar to

FIG. 3. Comparison of the effect of trypsin on the hemolytic activity of egg and L Sendai. The curves were obtained from data identical to that presented in Fig. 2. Hemolysis caused by egg Sendai (●) and L Sendai (○) at 188 hemagglutinin units per ml was plotted as a function of trypsin concentration.

FIG. 4. Effect of sonic treatment on the hemolytic activity of egg and L Sendai and effect of trypsin treatment of the sonically treated viruses on their hemolytic activities. Egg Sendai at 32 hemagglutinin units (HA) per ml and L Sendai at 512 HA per ml were sonically treated as described in Materials and Methods. After various periods of time, a sample from each preparation was withdrawn and divided into two portions. One-half was treated with trypsin at a final concentration of 0.0004% for 6 min at 36 C, and the other half remained untreated. The hemolytic activity was measured as described in Materials and Methods. Symbols: (●—●) sonically treated egg Sendai, (○—○) sonically treated egg Sendai followed by trypsin treatment, (●—●) sonically treated L Sendai, (○—○) sonically treated L Sendai followed by trypsin treatment.

that obtained with egg Sendai, but not to that obtained with L Sendai before trypsin treatment. Although figures are omitted for simplicity, when the order of treatment of egg and L Sendai with sonic treatment and trypsin was reversed, almost the same patterns as before were obtained in either case. These results may indicate that as far as hemolytic activity is concerned L Sendai has been converted to egg Sendai by trypsin treatment. With regard to the HA titer of egg and L Sendai, a twofold increase was obtained by sonic treatment for 30 sec to 3 min, the same as the original titer for 5 min, a twofold decrease for 10 min, and no activity for 20 min, respectively. The trypsin treatment under the given conditions did not alter the HA activity at all. From the

facts, an apparent discrepancy between the principles responsible for the hemolytic activity and HA activity has been revealed by sonic treatment, and the enhancement of hemolysis could not relate to the increase in the HA titer.

Effect of freezing and thawing on the hemolysis of egg and L Sendai. It was reported that the hemolysis of egg-grown Sendai (5, 11), mumps (1), and Newcastle disease (2) viruses was enhanced by freezing and thawing. Therefore, an experiment was carried out to see whether this treatment induces the enhancement of the hemolysis of L Sendai.

The fresh harvests of egg and L Sendai in the test tubes were subjected to one to six cycles of freezing and thawing. After every treatment two tubes of each were withdrawn and divided into two portions, one of which was further treated with trypsin at a concentration of 0.0004% for 6 min at 36 C, and all samples were measured for hemolytic activity as described.

Table 1 shows the results. An enhancement of the hemolysis of egg Sendai was evidently shown by freezing and thawing. The maximum enhancement was obtained after three cycles of freezing and thawing, but further treatments caused a gradual decrease. Although some increase was obtained with L Sendai by freezing and thawing,

TABLE 1. Effect of freezing and thawing on the hemolysis of egg and L Sendai^a

Freezing and thawing cycles	Hemolysis (OD ^b at 575 nm)			
	Egg Sendai (16 HA per ml)		L Sendai (1,024 HA per ml)	
	Before trypsin	After trypsin	Before trypsin	After trypsin
0	0.120	0.108	0.225	0.918
1	0.202	0.201	0.230	0.924
2	0.303	0.305	0.239	0.938
3	0.388	0.391	0.259	0.962
4	0.340	0.339	0.282	1.116
5	0.308	0.310	0.295	1.200
6	0.304	0.300	0.288	1.198

^a Samples of either egg or L Sendai were added to the test tubes and subjected to one to six cycles of freezing and thawing. At the end of every treatment, two tubes of each of the viruses were withdrawn and divided into two 0.3-ml portions. One-half was treated with trypsin at a final concentration of 0.0004% for 6 min at 36 C, and the other half remained untreated. Hemolytic activity was measured as described. Hemagglutinin titer was also measured with each sample; HA = hemagglutinin units.

^b Optical density.

TABLE 2. *Hemolysis of L Sendai released from infected L cells treated with trypsin^a*

Time of trypsin treatment after infection (hr)	Hemolysis (OD ^b at 575 nm)
None	0.018
3	0.015
10	0.015
20	0.150
38	0.320

^a At the times indicated, the monolayer cultures of L cells infected with egg Sendai were subjected to pulse treatment with trypsin as described. The released virus was collected at 48 hr after infection, purified, suspended in phosphate-buffered saline to yield 128 hemagglutinin units per ml, and tested for hemolytic activity.

^b Optical density.

the degree of increase was far less than that obtained with egg Sendai. Changes in HA activity were not demonstrated in either virus in this experiment. A great increase in the hemolytic activity of L Sendai was induced by the successive trypsin treatment, whereas significant increase could not be obtained with egg Sendai. In performing the freezing and thawing experiments, difficulty was encountered in reproducibility, and a typical pattern of the alteration of the hemolytic activity of egg and L Sendai by freezing and thawing could not be demonstrated. However, the following facts were constantly observed: freezing and thawing could induce a great increase in the hemolytic activity of egg Sendai but only a slight increase in that of L Sendai. The enhancing effect of trypsin on hemolysis was conspicuous with L Sendai, but a decreasing effect was usually obtained with egg Sendai. It is clear from the results that a mechanism of enhancement of the hemolytic activity of L Sendai by trypsin differs from the one which enhances the same activity of egg Sendai by freezing and thawing.

Attempt to obtain hemolytic L Sendai from the infected L cells. If the masking substance is located on the infected L cell surface and taken up by the progeny virus, trypsin treatment of the infected cells might remove such a substance, and the progeny virus release therefrom would be hemolytic.

L cells were treated with trypsin at 3, 10, 20, and 38 hr, after infection with egg Sendai and then incubated in the medium containing soybean trypsin inhibitor. The viruses released from each were collected at 48 hr after infection and tested for hemolytic activity.

Table 2 shows that, with trypsin treatment at 38 hr, the cells released highly hemolytic virus. A less highly hemolytic, but nevertheless signifi-

cantly hemolytic, virus was released from the cells treated at 20 hr, which is 4 hr before the mature virus starts to release (8). The trypsin effect was abolished if the cells were treated at any time before 10 hr after infection.

DISCUSSION

Apparent lack of hemolytic activity in egg-grown Sendai virus after single passage into tissue culture cells, that was attributed to a host-controlled modification, has been reported (6, 8-10).

The results obtained in the present study have clearly shown that hemolytic activity was markedly enhanced by the enzymatic action of trypsin on L Sendai, indicating that the apparent lack of hemolysis with L Sendai preparation is not due to substantial loss of the hemolytic activity with L Sendai virion but to masking by some inhibitory substance which can be removed by suitable treatment with trypsin as described. On the contrary, egg Sendai was highly hemolytic, and the enzymatic enhancement was barely demonstrable, suggesting that egg Sendai is mostly, if not entirely, composed of fully hemolytic particles because of the inducibility of the trypsin enhancement.

That L Sendai without trypsin treatment always showed a slight, but definite, degree of hemolysis (Fig. 1, 2, and 4) is a matter of interest. It is unlikely that L Sendai preparation is merely a mixture of the particles with substantial loss of the hemolytic activity and the ones with fully hemolytic activity (e.g., egg Sendai) because hemolysis of L Sendai was greatly enhanced by the trypsin treatment, whereas that of fully hemolytic egg Sendai was not enhanced by the same treatment. Thus, it seems that the L Sendai preparation is composed largely of the trypsin-inducible hemolytic particles, and the fully hemolytic particles, if any, may be a minor part of it.

As for the structure with which the hemolytic activity is associated, the experiments with sonic treatment in the present study are very suggestive (Fig. 4). Sonic treatment of egg Sendai greatly enhanced its hemolysis, and enhancement reached the maximum level after 3 min. Longer treatment decreased the hemolysis. Egg Sendai virion may be functionally composed of multiple smaller hemolytic units. Sonic treatment for an adequate time may release them from the virion as a functionally active form, but a longer treatment may inactivate them. The data presented in Fig. 4, therefore, may be understood as the sum of these dual opposite effects. It has been reported by Hosaka (7) that sonic treatment of egg-grown Sendai virus yielded smaller envelope fragments

with hemolytic activity. He has also claimed that the cooperation of a certain number of neighboring spikes may be required for hemolysis to occur. Although the morphological structure responsible for the hemolytic activity of Sendai virus has not yet been known, it may be composed of a certain number of morphological units. In the case of L Sendai, enhancement of hemolytic activity reached the maximum level after very short periods of sonic treatment (30 sec), and the degree of enhancement was very limited (Fig. 4). These facts might indicate that the hemolytic unit of L Sendai occupies a larger area than that of egg Sendai and that the number of such units per virion is far smaller as compared with egg Sendai. Sonic treatment may readily destroy hemolytic activity and release only a very limited number of the hemolytic units from the L Sendai virion, even under the optimal condition. However, the fact that hemolysis of L Sendai could markedly be enhanced by trypsin treatment suggests that the L Sendai virion is equipped in itself with a large number of hemolytic units and more likely with morphological units, in occult form, which can be activated by the trypsin treatment. This idea is supported by the fact that when L Sendai was treated first with trypsin and then subjected to sonic treatment for various lengths of time, the pattern of the alteration of hemolysis became similar to that obtained with egg Sendai by sonic treatment (Fig. 4).

In the foregoing paper (4), the author has operationally proposed a masking substance as the target of trypsin action to explain the inefficiency of the infectivity of L Sendai for L cells. The masking substance may be supplied from the host cell to the surface of the L Sendai virion in the course of its multiplication, probably by maturation, and it inhibits the infectivity for L cells by some unknown mechanism. This hypothesis also can be adopted favorably to explain the given phenomenon under study. From this point of view, L Sendai preparation may consist of the virus particles with varying amounts of the masking substance. Thus the fully hemolytic particle in the L Sendai preparation may be an extreme freed from the masking substance and it may behave as egg Sendai. The trypsin-inducible hemolytic particle may be saturated with or partially covered with the masking substance and be converted to the fully hemolytic particle after trypsin treatment. The existence of the masking substance is strongly suggested by the experiment, which shows that the progeny virus released from the trypsin-treated infected L cells was highly hemolytic. The trypsin treatment could remove the masking substance from the L cell

surface on which it had been located. However if the same treatment occurred at earlier times of infection, hemolytic virus could not be recovered, suggesting that the masking substance is synthesized continuously, even in the infected cells, after removal by trypsin.

Little has been known about the role of the hemolytic activity of Sendai virus in the infection processes. It seems worthwhile, therefore, to recall that L Sendai is able to adsorb to and elute from red blood cells and also to adsorb to L cells, but neither lyses the red blood cells nor penetrates L cells (3, 8). In addition, the present study has revealed that the optimal condition for trypsin treatment of L Sendai (0.0004%, for 6 min at 36 C) for the induction of the maximal enhancement of hemolytic activity was the same as that of infectivity for L cells (4), suggesting that masking of the infectivity and the hemolytic activity of L Sendai is caused by a common mechanism and the masking substance responsible for the respective phenomena is the same entity. With Sendai viruses grown in HeLa, FL, and chick embryo cells, apparent lack of hemolytic activity was inevitably accompanied by lack of infectivity for the corresponding host cells (*unpublished data*). These seem to have disclosed a similarity between the penetration process in the L cell system and the lytic process in the red blood cell system. However, a definite conclusion should be drawn after more detailed comparative studies on the restoration processes of infectivity and hemolysis are made.

A previous paper (4) disclosed differences in the densities of egg and L Sendai (1.174 and 1.207, respectively) due to a modification by the host cells. This change may have been related to the lipid content of the virus envelope. Because no significant change in the densities of egg and L Sendai occurred after exposure to trypsin under the optimal conditions described for the induction of the hemolytic activity of L Sendai, the lipid responsible for lowering the density of egg Sendai may not be essential for the hemolysis of Sendai virus; the variations of Sendai virus in density and hemolysis are uncoupled host-controlled modifications.

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