JOURNAL OF VIROLOGY, Nov. 1971, p. 619-629
Vol. 8, No. 5

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

I. Restoration of the Infectivity for L Cells by Direct Action of Trypsin on L Cell-Borne Sendai Virus

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Received for publication 19 July 1971

Sendai virus grown in fertile eggs (egg Sendai) infects L cells in which the synthesis of L Sendai (grown in L cells) occurs by the one-step mechanism. L Sendai is not infectious for L cells when tested by the tube titration method although it is infectious for chick embryos. When L cells infected with egg Sendai were dispersed by trypsin and plated on a monolayer culture of L cells, the viral agents spread to the adjacent recipient cells in which the synthesis of L Sendai occurred. The newly infected L cells became infectious for L cells again by trypsin treatment. Kinetic experiments suggested that the target of trypsin is the mature virus, of L Sendai nature, just budding from the L-cell surface. By using an immunofluorescent cell-counting technique, recovery of the infectivity of L Sendai for L cells due to a direct enzymatic action of trypsin was demonstrated. Under the optimal condition, the infectivity increased 1,000-fold for L cells and 10-fold for chick embryos, and both the titers could favorably be compared. No increasing effect of trypsin was observed on the infectivity of egg Sendai. Density centrifugation studies revealed a difference between egg Sendai and L Sendai in the density. Trypsin treatment which induced the maximal enhancement of L Sendai infectivity did not affect both the densities, showing that variations of Sendai virus in the infectivity for L cells and in the density are independent types of host-controlled modification.

Host-induced modifications, made by acquiring structural materials directly from host cells, are rather commonly observed among myxoviruses (4, 14, 15, 18, 27). In previous papers, we reported that Sendai virus grown in L cells (L Sendai) was markedly different from the one grown in the allantoic cavity of fertile eggs (egg Sendai). Egg Sendai showed both a characteristic cytopathic effect (CPE) on L cells with production of L Sendai progeny and hemolytic activity. In contrast, L Sendai did not show CPE on L cells, any sign of the synthesis of viral antigens in L cells, or hemolytic activity although it retained the infectivity for chick embryos (10, 11). The failure of growth of L Sendai in L cells was due to its being incapable of penetrating L cells, whereas adsorption occurred to an extent similar to that of egg Sendai (8, 10). Since conversion of egg Sendai to L Sendai and reversion of L Sendai to egg Sendai occurred in a single growth cycle in the respective hosts, the observed phenomenon was understood as a host-controlled variation.

The present paper is concerned with the findings that when the monolayer culture of L cells primarily infected with egg Sendai is dispersed by trypsin and plated on the fresh monolayer culture of L cells, it spreads the infectious agents to the adjacent cells and produces a macrovisible hemadsorption focus. Several experiments to elucidate this phenomenon were carried out, and a direct effect of trypsin on L Sendai virion for restoration of the infectivity for L cells will be revealed. Conditions in which the maximal infectivity of L Sendai is induced by trypsin treatment are also determined.

MATERIALS AND METHODS

Virus. The Fushimi strain of Sendai virus was grown in the allantoic cavity of 10-day-old chick embryos from a 10^6 dilution of seed virus, and the allantoic fluid was harvested after incubation for 72 hr at 36 C (egg Sendai). L cell-borne Sendai virus (L Sendai) was recovered from the tissue culture fluids of the standard passage of infected L cells as will be described in detail below. Both viruses were purified partially by differential centrifugations at 4,000 × *g* for 15 min and 43,000 × *g* for 30 min. The virus pellets were

suspended in a phosphate-buffered saline (PBS), *p*H 7.2, and clarified by a centrifugation at 4,000 × *g* for 15 min which was followed by filtration through a membrane filter (type HA; Millipore Corp., Bedford, Mass.). It should be emphasized that L Sendai thus prepared did not contain even a trace of egg Sendai.

Virus assay. The infectivity for chick embryos was determined by inoculating 10-day-old chick embryos with serially 10-fold diluted Sendai virus. Five eggs were used per dilution, and the 50% egg infectious dose (EID_{50}) was calculated by the Reed and Muench method. The infectivity for L cells was determined either by titration in the monolayer culture of L cells or by an immunofluorescent cell-counting technique which has been established in this system (13). In the former, the 50% tissue culture dose (TCD_{50}) was calculated on the basis of the appearance of CPE.

Tissue culture. L cells were seeded into either test tubes (10 by 100 mm) or 60-mm petri dishes in a growth medium which consisted of 0.1% yeast extract, 0.5% lactalbumin hydrolysate in Earle's balanced salt solution with 0.45% glucose (YLE) and 10% bovine serum. Petri dish cultures were incubated at 36 C in 5% CO_2 in humidified air. For virus infection, a maintenance solution which consisted of YLE with 2% heated horse serum (MS) was substituted for the growth medium.

Immunofluorescent staining. Cover slip (6 by 30 mm) cultures of L cells were made in petri dishes (13). Each cover slip with complete monolayer cells received 0.05 ml of test samples. After incubation for 1 hr at 36 C, the cover slips were immersed in new petri dishes containing 5 ml of MS, and incubation was continued in a CO_2 chamber. At the indicated times, the cover slips were removed from the petri dishes, washed in PBS, dried, fixed with acetone, and stained with fluorescent antibody by the direct method.

Antiviral serum. A 1-ml amount of the partially purified egg Sendai [2,000 hemagglutinin units (HAU)/ml] was inoculated intravenously twice a week into a rabbit. A total of seven successive injections was made, and the animal was bled 1 week after the last injection. A portion of the antiserum was used for conjugation of gamma globulin with fluorescein isothiocyanate.

Treatment of Sendai virus with trypsin. To 0.3 ml of virus suspension was added 0.1 ml of appropriately diluted crystalline trypsin in PBS. After incubation in a water bath at 36 C, the mixtures were immediately chilled and diluted 1,000-fold in cold MS to reduce the enzyme level sufficiently. Otherwise, as indicated in the text, 0.1 ml of soybean trypsin inhibitor was added to the reaction mixtures.

Equilibrium density gradient centrifugation. A linear potassium tartrate density gradient in PBS (60 to 3%, w/v) was prepared by the method proposed by McCrea et al. (16). Virus samples were loaded on top of the gradients (4.5 ml), and centrifugation was carried out in a Spinco SW 39 head at 39,000 rev/min for 60 min at 4 C. After centrifugation, fractions were collected by piercing the bottom of the centrifuge tube with a needle.

Trypsin and soybean trypsin inhibitor. In the experiments specified, crystalline trypsin (thrice crystallized; C. F. Boehringer & Soehne GmbH, Manheim, Germany) and crystalline soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) were used. They were diluted by PBS, divided into small test tubes, and stored in a deep freezer at −80 C until desired.

RESULTS

Serial passage of L cells infected with Sendai virus by trypsin treatment. As reported previously, L Sendai did not infect L cells although it did so chick embryos. Criteria for loss of the infectivity for L cells were based on lack of the CPE, hemagglutinin (HA) production, and hemadsorption. After many attempts to infect L cells with L Sendai, it was eventually found that, when L cells which had been infected with egg Sendai were dispersed by trypsin and plated on a fresh monolayer culture of L cells, CPE appeared in the recipient cells around the inoculated cells. The CPE became manifest at about 10 to 12 hr after inoculation of the cells, and immunofluorescent staining detected the synthesis of viral antigens. The newly infected, virus-synthesizing cells were again made infectious for L cells by trypsin treatment. The virus-synthesizing cells then could easily be transferred successively, and the following procedure was established to passage the infectious state of L cells on the monolayer culture of L cells (standard passage).

A petri dish culture of L cells was infected with 5 ml of egg Sendai at a multiplicity of 10 EID_{50} per cell in MS. After adsorption for 1 hr, the culture was washed twice with Hanks solution and reincubated in 5 ml of fresh MS at 36 C. A characteristic CPE appeared on the next day, and virus yields in the fluids reached maximum at about 72 hr after infection. The cultures were then washed once with PBS, 0.05% trypsin (Nutritional Biochemicals Corp., 1:300) in PBS was added for 1 min at 36 C, the trypsin was aspirated, and incubation continued for another 5 min. The detached cells were dispersed by gentle pipetting in a small amount of MS and diluting. More than 2×10^5 cells in 5 ml of MS were inoculated on a fresh monolayer culture of L cells in a petri dish. Almost all of the cells could be infected under this condition. Serial passages of the infected cells were made at intervals of every 2 days, and the cells at infectious state have been maintained over a period of 100 passages.

Figure 1a shows the immunofluorescent feature of the microfoci produced by inoculating the cover slip culture with the trypsinized cells from the 50th standard passage. This can be compared with Fig. 1b where the cells were infected with egg Sendai at a low multiplicity. The immunofluorescence was almost confined to a single cell

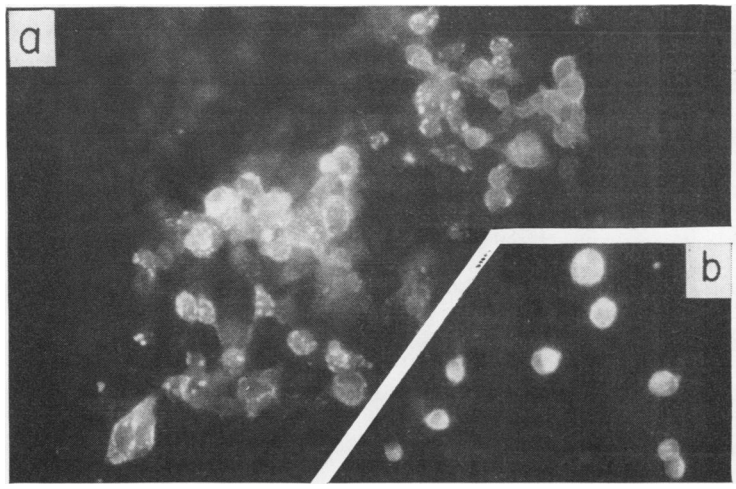

FIG. 1. *Immunofluorescence features of L cells produced by the trypsinized, infected L cells and by egg Sendai. (a) Two-day-old infected L cells in petri dish at the 50th standard passage were dispersed by trypsin, and 0.05 ml of the suspension containing 1,000 cells was inoculated on the cover slip culture of L cells. Immunofluorescent staining was made at 48 hr after inoculation of the infected cells. Fluorescent-positive cells appear focuswise, and three foci are evidently shown. (b) The cover slip culture of L cells was infected with egg Sendai at a multiplicity of 0.1 EID_{50} per cell and immunofluorescent staining was made as in (a). No tendency of spread of the fluorescence from singly infected cell to the adjacent cells is observed.*

and, as time elapsed, no tendency of spreading of the fluorescence to the adjacent cells was observed.

Properties of the virus recovered from the standard passage. The culture fluid of the standard passage usually contained more than $2^{8.0}$ HAU/ml at 72 hr after infection. An experiment was undertaken to see whether the virus recovered from the standard passage still retained the property comparable with that of the virus produced after a single growth cycle of egg Sendai in L cells. In this experiment, the HA activity and the infectivity for chick embryos and for tube cultures of L cells served as criteria of the biological activity of egg Sendai and L Sendai. The viruses tested were as follows: (i) the egg-passaged line of Sendai virus (egg Sendai), (ii) a single passage of egg Sendai in L cells (L Sendai-1), (iii) the culture fluid of the 50th standard passage (L Sendai-50), and (iv) a single passage of L Sendai-50 in chick embryos (L Sendai-50-egg-1). For preparation of L Sendai-1, care was taken to remove the residual contaminant of egg Sendai by pulse treatment of the culture with the antiserum after virus adsorption. All tested viruses were diluted in MS to yield 1,280 HAU/ml.

Table 1 shows the results. L Sendai-1 and L Sendai-50 did not reveal the infectivity in the L-cell tube titration, whereas they did for chick embryos. However, the EID_{50}:HA ratio was as low as $10^{4.5}$ with L Sendai-1 and $10^{4.3}$ with L Sendai-50, whereas that of egg Sendai was $10^{6.2}$, being in accord with the previous results (10, 11). The virus obtained after a single passage of L Sendai-50 into chick embryos (L Sendai-50-egg-1) restored the infectivity for L cells to a comparable level with egg Sendai. These results showed that the property of L Sendai could stably be maintained during numerous passages in L cells.

Demonstration of cell-to-cell infection in the standard passage. This experiment was done to study the mechanism of spreading of the viral agents from the trypsinized infected cells to the recipient L-cell monolayers. L cells at the 55th standard passage were trypsinized on the 2nd day after infection, diluted by MS to yield 1,000 to 2,000 cells per 0.05 ml, and plated on the cover slip cultures. The immunofluorescent staining was made at 36 and 72 hr after cell inoculation, and frequency distribution of the number of the fluorescent cells in the foci was determined by counting 500 foci with respective preparations. The results are illustrated in Fig. 2, showing that more than 70% of the foci were composed of about 16 to 24 virus-synthesizing cells. Apparently, the curve did not show the normal distribution pattern, which suggests that some of the foci were derived from multiple cells. No significant difference in the distribution pattern was observed as the incubation period was prolonged from 36 to 72 hr. Since the virus from the standard passage was not infectious to L cells, this result seemed to suggest that spreading of the

TABLE 1. *Comparison of the infectivity of egg and L Sendai for chick embryos and L-cell tube cultures*

Virus[a]	Virus titer[b]			Ratio of EID_{50} to	
	HAU/ml	EID_{50}/ml	TCD_{50}/ml	HAU	TCD_{50}
Egg Sendai	1,280	$10^{9.3}$	$10^{4.5}$	$10^{6.2}$	$10^{4.8}$
L Sendai-1	1,280	$10^{7.6}$	$<10^{-0.5}$	$10^{4.5}$	$>10^{8.1}$
L Sendai-50	1,280	$10^{7.4}$	$<10^{-0.5}$	$10^{4.3}$	$>10^{7.9}$
L Sendai-50-egg-1	1,280	$10^{9.4}$	$10^{4.5}$	$10^{6.3}$	$10^{4.9}$

[a] Egg Sendai, virus grown in fertile eggs; L Sendai, virus grown in L cells; L Sendai-1, single passage of egg Sendai in L cells; L Sendai-50, culture fluid of the 50th standard passage; L Sendai-50-egg-1, single passage of L Sendai-50 in chick embryos.

[b] HAU, hemagglutinin units; EID_{50}, 50% egg infectious dose; TCD_{50}, 50% tissue culture dose.

viral agents occurred directly from the trypsinized seed cells to the adjacent recipient cells.

Detection of the foci by hemadsorption. The foregoing results suggested that the immunofluorescent foci could also be detected by hemadsorption without agar overlay. In fact, hemadsorption foci appeared first at about 12 hr after inoculation of the trypsinized cells from the standard passage. Minute but discrete hemadsorption foci were formed at 72 to 96 hr, and they could easily be detected macroscopically. A quantitative study revealed a linear relationship between the number of hemadsorption foci and that of the cells seeded. However, the efficiency fluctuated considerably from one experiment to another, ranging from 30 to 95%, which might be caused by the existence of various numbers of uninfected cells among the cell population in every standard passage.

Time course of appearance of the trypsin-inducible infectious state of L cells after infection with egg Sendai. It was of interest to pursue the time course for L cells infected with egg Sendai to acquire the infectivity for L cells by trypsinization in direct comparison with the time course of the formation of infectious virus for chick embryos. Replicating tube cultures of L cells were infected with egg Sendai at a multiplicity of 10. The washing procedure was the same as described for the preparation of L Sendai-1. At appropriate intervals, the tubes were taken out and divided into two parts. After being washed with PBS, half of the tubes were dispersed by trypsin; the cells were plated on the monolayer culture of L cells, and the resulting hemadsorption foci were counted on the 3rd day thereafter. With the remaining half, the whole culture was broken up by four cycles of freezing and thawing. This was followed by centrifugation at 4,000 × *g* for 15 min, and the supernatant fluid was measured for infectivity for chick embryos (Fig. 3). The hemadsorption foci began to form between 11.5 and 14 hr after infection, this time being well

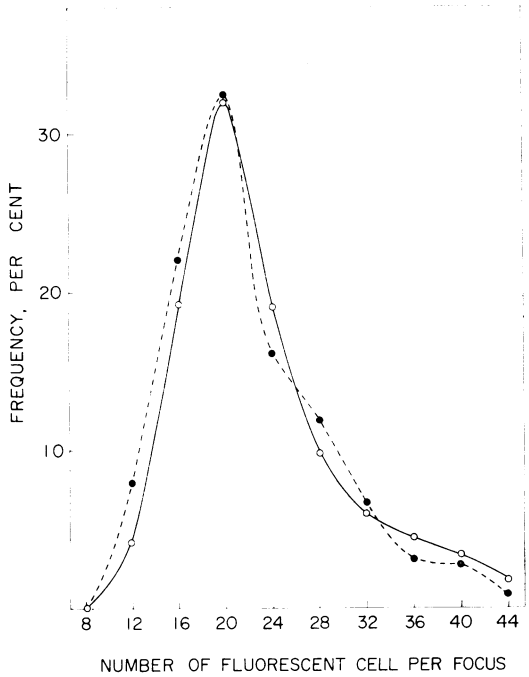

FIG. 2. *Frequency distribution of the number of fluorescent cells in the foci produced by trypsinized, infected L cells. The cover slip cultures of L cells were inoculated with 2-day-old L cells at the 55th standard passage after trypsinization as described in Fig. 1a. Immunofluorescent staining was made at 36 hr (○) and 72 hr (●) after inoculation of the infected cells, and frequency distribution of the number of fluorescent cells in the foci was determined.*

corresponded to the one at which the mature virus starts to form. No hemadsorption foci were formed when the cells, at 29.5 hr after infection, were collected by vigorous pipetting instead of trypsinization and plated on the monolayer culture.

Effect of freezing and thawing on the trypsin-induced infectious state of L cells. The foregoing

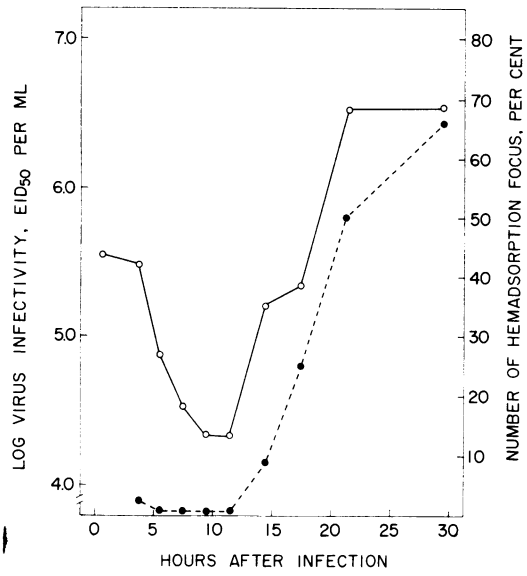

FIG. 3. *Time course of increase of the trypsin-inducible infectious cells in comparison with virus growth after infection of L cells with egg Sendai. The tube cultures of L cells were infected with egg Sendai at a multiplicity of 10 EID_{50} per cell and were divided into two portions as described in the text. At the indicated times, the cells from the one half were dispersed by trypsin, diluted into MS, and plated on the monolayer culture of L cells in a petri dish. Hemadsorption was made at 72 hr after infection, and the efficiency of the formation of hemadsorption foci was calculated* (●). *The remaining tubes were frozen and thawed four times, and the cell debris was removed by centrifugation at 3,000 rev/min for 5 min. The resulting supernatant fluids were inoculated into chick embryos and EID_{50} was determined* (○).

results posed these questions. Why do the cells, up to 11.5 hr after infection, not become infectious for L cells by trypsin treatment? What is the nature of the trypsin-inducible infectious state? To answer these questions, the effect of freezing and thawing on the trypsin-induced infectious cells was studied.

The cells at the 74th standard passage were dispersed by trypsin 2 days after infection and diluted fourfold with MS. Each dilution was divided into two parts. One half was disrupted by freezing and thawing, and the other half remained untreated. A 1-ml portion of each was inoculated into a petri dish culture. The hemadsorption foci were counted on the 3rd day, and the virus yields were determined by measuring HA titer of the culture fluid. As a control, the cells from the standard passage were dispersed by means of vigorous pipetting instead of trypsinization and were then treated as described above (Table 2). The growth of virus was significant only in the dishes which received the cells treated with trypsin. Freezing and thawing did not affect the virus yield markedly. Typical hemadsorption foci were obtained in the dishes inoculated with the intact trypsinized cells. Freezing and thawing abolished the formation of the typical hemadsorption foci. Regardless of the cell treatment, hemadsorption occurred in a diffuse area when a large number of cells (e.g., 10,000) were plated which could easily be differentiated morphologically from the typical foci. Although a small number of discrete hemadsorption foci were apparently obtained by freezing and thawing of the cells, with or without trypsin treatment, they were microscopically shown to be aggregates of the disrupted cells.

These results would indicate that an integrated form of the trypsinized cell is necessary to produce the hemadsorption focus. Since freezing and thawing of such cells did not affect the virus yield significantly, the number of the trypsin-induced infectious units seemed unaltered by this treatment.

Inhibition of the focus formation by the antiviral serum. Considering that paramyxovirus simian virus 5 is closely related to Sendai virus and matures through budding from cell surfaces (3) like other myxoviruses (5, 9, 19, 22), the results obtained in Fig. 3 and Table 2 seem to suggest that the real target of trypsin action is the mature virus budding from the infected L-cell surface. If this is the case, transfer of the infectious agents from the trypsin-induced infectious cells to the adjacent cells would be prevented by the antiserum against Sendai virus.

The cells from the standard passage were dispersed by trypsin, and a 5-ml sample of MS containing 300 cells was inoculated on the monolayer culture of L cells. At indicated times, 0.5 ml of the antiserum was added to the culture at a final dilution of 1:100. After incubation for 24 hr, the antiserum was removed; the monolayer was washed with Hanks solution and fed with fresh MS. At 72 hr after cell inoculation, the hemadsorption foci were counted (Table 3).

The antiserum completely inhibited the formation of the hemadsorption foci when added at earlier times after cell inoculation. As the time of addition of the antiserum was delayed, the inhibition became less pronounced although some residual effect of the antiserum was observed even at 28 hr under the given condition.

Direct effect of trypsin on the infectivity of egg and L Sendai. The facts mentioned above (Fig. 3, Tables 2, 3) are all compatible with the idea that the target of trypsin is the mature virus. It has

TABLE 2. *Effect of freezing and thawing on the formation of hemadsorption foci*

Treatment for cell dispersion	No. of cells inoculated per dish	Treatment of cells before inoculation			
		With freezing and thawing		Without freezing and thawing	
		Hemagglutinin yield per ml	No. of foci per dish	Hemagglutinin yield per ml	No. of foci per dish
Pipetting	10,000	4	Diffuse[a]	6	Diffuse[a]
	2,500	<1.5	24	<1.5	20
	625	<1.5	12	<1.5	9
	156	<1.5	4	<1.5	5
Trypsin	10,000	64	Diffuse[a]	75	Numerous[b]
	2,500	8	25	24	Numerous[b]
	625	2	18	4	260
	156	<1.5	6	<1.5	80

[a] Hemadsorption occurred in diffuse area but no discrete foci appeared.
[b] Numerous but discrete foci appeared.

been reported, however, that treatment of Sendai virus with trypsin (Difco, 1:250, 0.1%) readily released both the HA and neuraminidase from the viron (17). In addition, in my preliminary observations, the infectivity of egg Sendai was reduced 1,000-fold by the same treatment with trypsin as that used for the cell dispersion hitherto described, and morphological integrity of the virion was lost simultaneously. Accordingly, it was thought that if trypsin acts directly on the free L-Sendai virion to activate its infectivity for L cells, the optimal concentration would be very low.

To ensure the enzymatic action of trypsin, the experiments were conducted hereafter with crystalline trypsin. Egg and L Sendai were treated with various concentrations of trypsin. Immediately after incubation for 10 min, the virus-trypsin mixtures were diluted and assayed for the infectivity for L cells by the immunofluorescent cell-counting technique (Fig. 4).

In the range of the concentrations used, an increase in the infectivity of L Sendai was noted. Evidently, there was an optimal concentration to induce the maximal infectivity. Trypsin at a final concentration of 0.0004% caused about a 1,000-fold increase. In contrast, there was no increasing effect of trypsin on egg Sendai. The infectivity of both viruses became rapidly inactivated at concentrations over 0.006%. It should be noted that a very small fraction in the L Sendai preparation was infectious for L cells without trypsin treatment, which could be detected only by the immunofluorescent cell-counting method but not by the ordinary tube titration method.

Inhibition of the trypsin action by soybean trypsin inhibitor. To verify that the restoration of the infectivity of L Sendai was truly mediated by the enzymatic action of trypsin, the effect of soybean trypsin inhibitor was studied on the induction of a specific inhibition of the trypsin action as described above.

TABLE 3. *Effect of the antiserum against Sendai virus on the formation of hemadsorption foci*

Time of addition of antiserum after infection (hr)	No. of cells inoculated per dish	No. of hemadsorption foci per dish
0	300	0
1	300	0
7.5	300	31
20	300	56
28	300	92
No serum	300	231

An equimolar concentration of the inhibitor to trypsin (0.0004%) was added to the reaction mixtures of trypsin and L Sendai either at the beginning or at the end of a 10-min incubation period at 36 C. In a control experiment, the reaction mixture was diluted in cold MS at the end of trypsin treatment to reduce the enzyme level below that shown to affect the infectivity.

As a result, the addition of the inhibitor to the reaction mixture at the beginning of the incubation inhibited the trypsin action completely.

Time of maximal trypsin action. It became evident from the preceding results that the trypsin action was immediately halted by soybean trypsin inhibitor. By using this inhibitor, therefore, the optimal reaction time of trypsin at a given concentration for induction of the maximal infectivity of L Sendai was determined. L Sendai was treated with trypsin at a final concentration of 0.0004% at 36 C. At the time indicated, the equi-

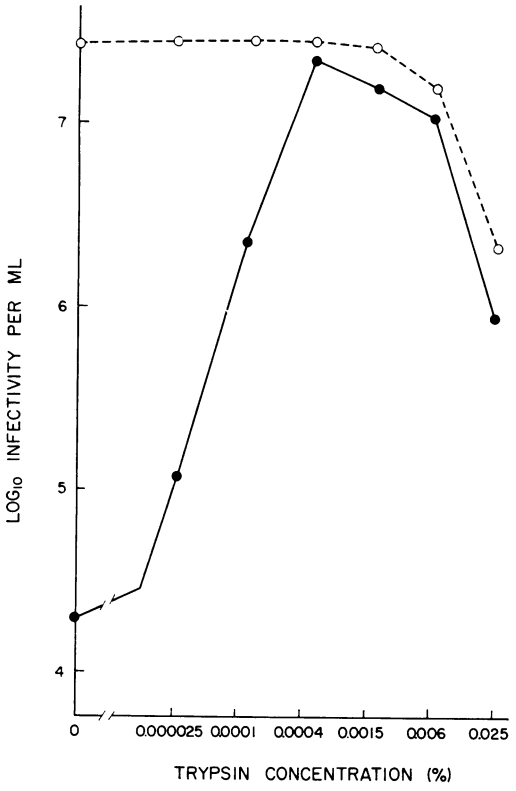

FIG. 4. *Effect of trypsin on the infectivity of egg Sendai and L Sendai. Egg Sendai (○) and L Sendai (●) were treated with various concentrations of crystalline trypsin for 10 min at 36 C. At the end of the incubation period, the virus-trypsin mixtures were diluted with cold MS. The infectivity for L cells was determined by the immunofluorescent cell-counting method.*

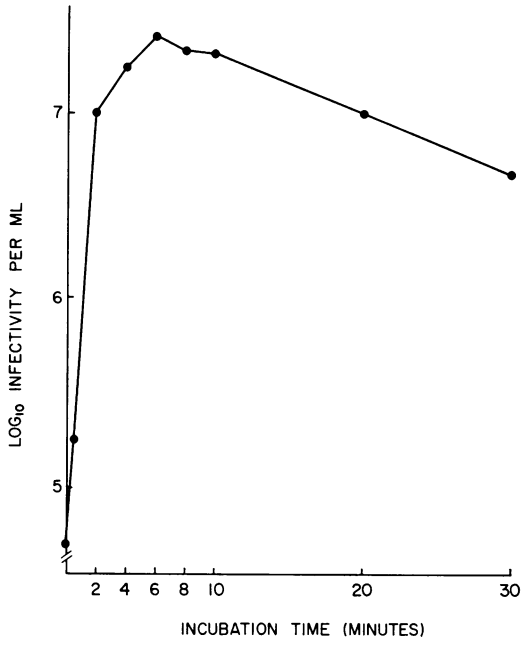

FIG. 5. *Kinetics of enhancement of the infectivity of L Sendai for L cells after exposure to trypsin. L Sendai was treated with crystalline trypsin at a final concentration of 0.0004% at 36 C. Soybean trypsin inhibitor was added to the reaction mixture at the time indicated. The infectivity assay was made by the immunofluorescent cell-counting method.*

molar concentration of the inhibitor was added to the mixture.

An increase of the infectivity started, without delay, after exposure to trypsin (Fig. 5). The rate of increase was very rapid up to 2 min. Maximal levels were reached between 4 and 10 min, and the infectivity decreased thereafter at a slower rate.

Comparison of the infectivity of L Sendai for L cells with that for chick embryos after trypsin treatment. Concerning the ratio of EID_{50} to HA titer, the value usually obtained was $10^{4.5}$ with L Sendai and $10^{6.0}$ with egg Sendai, showing some degree of defectiveness of L Sendai in the infectivity for chick embryos. Therefore, it was of interest to know whether trypsin treatment of L Sendai also results in the restoration of the infectivity for chick embryos.

The infectivity of L Sendai for chick embryos as well as for L cells was determined before and after treatment with trypsin at a final concentration of 0.0004% for 6 min at 36 C, the condition being suitable to induce maximal infectivity for L cells.

A 10-fold increase of the infectivity for chick embryos was obtained after trypsin treatment, whereas the increase of that for L cells was over 1,000-fold (Table 4). It is worth pointing out that the infectivity of L Sendai for L cells after trypsin treatment reached the level of the infectivity for chick embryos. If one assumes that 0.69 EID_{50} is equivalent to 1 EID_{100}, the titer for L cells is very close to that for chick embryos. None of the enhancing effect of trypsin was obtained on the HA titer under the given condition. Since the HA titer used in this experiment was 1:128, the ratio of EID_{50} to HA titer was calculated to be $10^{5.75}$ after trypsin treatment, a value being comparable with (if not exactly the same as) that of $10^{6.0}$ usually obtained from the egg Sendai preparation. Therefore, with regard to infectivity, L Sendai might have been activated almost in full by trypsin treatment.

Effect of pretreatment of L cells with trypsin on

TABLE 4. *Restoration of infectivity of L Sendai for L cells and for chick embryos after trypsin treatment*[a]

Treatment of L Sendai	Infectivity per ml for		Infectivity ratio (L cell/ chick embryo)
	L cell	Chick embryo	
None	$10^{4.30}$	$10^{6.85}$	0.003
Trypsin (0.0004%, 6 min, 36 C)	$10^{7.52}$	$10^{7.85}$	0.47

[a] Infectivity for L cells was determined by the immunofluorescent cell-counting method and that for chick embryos by egg titration (EID_{50}).

the restoration of the infectivity of L Sendai. Although the previous results have shown that the restoration of the infectivity of L Sendai resulted from a direct effect of the enzymatic action of trypsin on the L Sendai virion, it was still necessary to see whether trypsin affects L cells, thus restoring the infectivity of L Sendai.

The cover slip cultures of L cells were pretreated with either 0.0001 or 0.0004% trypsin for 6 min at 36 C. After removal of trypsin, the cells were infected with L Sendai in MS containing soybean inhibitor, and immunofluorescent cell counting was done. The pretreatment of L cells with trypsin did not result in the restoration of the infectivity of L Sendai at all, completely denying the possible involvement of the direct effect of trypsin on L cells in the phenomenon under study.

Equilibrium density gradient centrifugation of egg Sendai and L Sendai before and after trypsin treatment. It has been reported that treatment of Sendai virus with trypsin released its surface antigens with concomitant loss of the surface projections (17) and that extended treatment of influenza virus with protease resulted in a density change with concomitant loss of the surface morphology (1). Preliminary electron microscopic studies on L Sendai have shown that none of the morphological changes occurred after exposure to 0.0004% crystalline trypsin for 6 min at 36 C. An experiment was designed, therefore, to see whether change in the density occurred after treatment of L Sendai with trypsin at the concentration which induces the maximal restoration of the infectivity.

An equilibrium density gradient centrifugation of Sendai virus was made on a preformed gradient of potassium tartrate (16). Egg Sendai and L Sendai could be successfully banded by centrifugation in a short period of time at 100,000 × *g*. There was no change in the density by prolongation of the centrifugation time from 60 to 300 min.

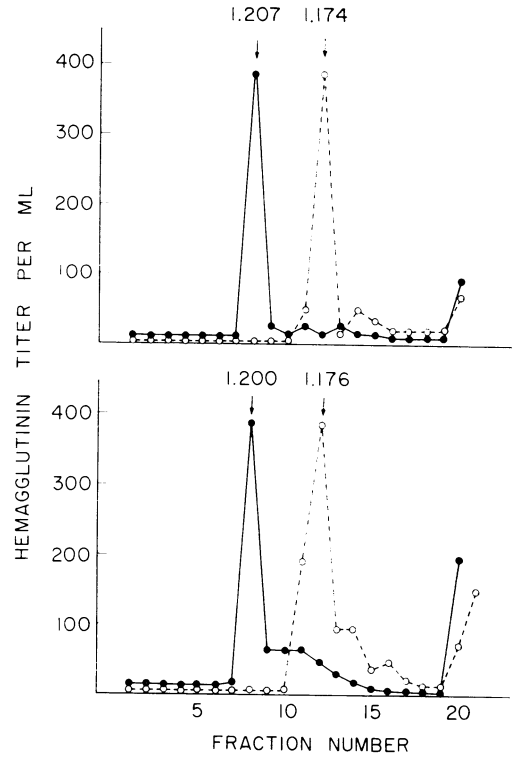

FIG. 6. *Sedimentation diagram of egg Sendai (○) and L Sendai (●) before (top) and after (bottom) trypsin treatment. The viruses were treated with 0.0004% crystalline trypsin for 6 min at 36 C, and the reaction was stopped by the addition of soybean trypsin inhibitor. The samples were loaded on the top of a linear potassium tartrate gradient (60 to 3%, w/v) and centrifuged for 60 min at 39,000 rev/min in a Spinco SW 39 head. The fraction was the top to the right.*

Egg Sendai and L Sendai showed different densities before exposure to trypsin, 1.174 and 1.207, respectively (Fig. 6). After exposure to trypsin, both egg Sendai and L Sendai were distributed rather widely toward lower densities. The peak fractions, however, were banded almost at the same position as those before exposure to trypsin. Although the data were omitted for brevity, the density change occurred after a single growth cycle in the respective hosts, showing a type of host-controlled modification as was the case in Newcastle disease virus (27).

DISCUSSION

Effect of proteolytic enzymes on enhancement of plaque size and number has been described by

several authors for enterovirus (28), reovirus (29), myxovirus (2, 20, 23), and vaccinia virus (6, 7). The effect of the proteolytic enzymes on the virus-cell systems seems manifold and may include the following: (i) the effect on the cell, (ii) the effect on the virus aggregates, (iii) the effect on the virus, (iv) the dual effect both on the cell and virus, (v) the effect on the course of the virus replication, and (vi) the effect on the interferon.

Enhancement of reovirus infectivity due to direct action of proteolytic enzymes on proteinaceous substance associated with the virion has been suggested (24, 26). More recently, the enhancing effect was shown to be attributed to the removal of the outer capsid by the enzyme treatment (25).

Enhanced production and serial passage of parainfluenza type 4 virus (20) and Sendai virus (12) in Vero cell cultures by incorporation of proteolytic enzymes in the culture fluid were reported. Similar enhancement by trypsin of the infectivity of parainfluenza type 3 virus in HEp-2 cells was also demonstrated (21). Enhancement by proteolytic enzymes of plaque formation by parainfluenza type 4 virus on Vero cell monolayers (20) and by Sendai virus on chick embryo cell monolayers (23) has been reported. In these studies, however, the authors did not work with the mechanism of the enhancement phenomenon.

To study the effect of the enzyme on a virus-cell system, the agar overlay technique seems unfavorable. Since continuous presence of the enzyme in the agar overlay media results in a continuous effect of the enzyme both on the cells and the virus, the conclusions drawn may be conjectural. In this respect, the Sendai virus-L cell system is advantageous to disclose a direct effect of trypsin on the virus for the restoration of the infectivity: (i) the growth of the virus in L cells occurs apparently by the one-step mechanism; (ii), therefore, the immunofluorescent cell-counting technique in the present system makes it possible to enumerate the infectious particles for L cells; and (iii) the use of soybean trypsin inhibitor immediately induces the inhibition of the trypsin action for the given phenomenon.

The present study has clearly shown an enhancing effect of trypsin on the infectivity of L Sendai for L cells. Obviously, there was an optimal condition (0.0004%, for 6 min, at 36 C) to induce the maximal enhancing effect. On the contrary, none of the enhancing effect was obtained by the pretreatment of L cells with trypsin. These results are in accord with the finding that Sendai virus-infected L cells became infectious for L cells by trypsin treatment only after the mature virus started to form (Fig. 3). A possibility that disaggregation of the clumps of L Sendai by trypsin treatment is responsible for the enhancement phenomenon is not likely because the treatment of L Sendai with trypsin at 0.0004% enhanced the infectivity 1,000-fold selectively for L cells and only 10-fold for chick embryos, while it did not affect the HA titer at all (Table 4).

Although the author did not work with chemicals other than trypsin, specificity of the enzymatic action of trypsin for the given phenomenon could be ascertained by an immediate inhibition of trypsin by soybean trypsin inhibitor. However, the mechanism of the enzymatic enhancement is still unknown. A possible explanation for the given phenomenon is that Sendai virus acquires an inhibitory substance in the course of its multiplication in L cells. Without evidence of the existence of such substance, the author is inclined to call it a "masking substance" for the sake of convenience. The masking substance may be localized on the surface of L Sendai. Treatment of L Sendai with a low concentration of trypsin may inactivate it functionally or remove it from the virion without affecting the morphological integrity and the infectivity of the virion. None of the enzymatic enhancement was observed with egg Sendai, suggesting that egg Sendai is not associated with the masking substance and is expressing its activity in full as far as the infectivity is concerned. Since the infectivity of L Sendai for chick embryos is higher than that for L cells, inefficiency of the infectivity of L Sendai due to the masking substance may be expressed differently by the kind of cell to be infected.

As has been pointed out in the text, the immunofluorescent cell-counting technique has revealed the existence of the particles in the L Sendai preparation, which are infectious for L cells without trypsin treatment (infectious L Sendai), in a proportion of 1 to 1,000 (Fig. 4). There is circumstantial evidence to indicate that the ability of the infectious L Sendai to infect L cells is not genetically determined. First, the infectivity of L Sendai has not been demonstrated by the ordinary tube titration method on L cells, where the CPE and the production of HA were employed as an indication of the infection. Second, a number of blind passages of L Sendai on L cells have failed to recover the transmissible agents. Third, the repeating trials to pick up a plaque former on the L-cell monolayers have not succeeded. In view of these facts, it may be thought that L Sendai preparation consists of the virus particles with various degrees of the masking substance. Thus, the infectious L Sendai may be either freed from or associated with the

substance to a lesser extent than the majority of the viruses. Upon infection with the infectious L Sendai, L cells will produce the viruses again with various degrees of the masking substance, in which the majority of the viruses are not infectious and only a small number of viruses are infectious for L cells. As a consequence of that, the infection of L cells with L Sendai will seemingly terminate after one-step growth.

Density centrifugation study revealed a difference between egg Sendai and L Sendai in the density distribution pattern. Egg Sendai may gather much host component, probably of lipid nature, which is not essential for the virion infectivity. There was no difference in the density of L Sendai before and after exposure to trypsin. This may indicate that the masking substance occurs in very small amounts, if it exists at all. In addition, the fact shows that density variation in Sendai virus is a type of host-controlled modification, supporting the work with Newcastle disease virus by Stenback and Durand (27), and suggests that variation in the density and the infectivity is an independent indication of host-controlled modification.

Change of Sendai virus in hemolytic activity has also been attributed to host-controlled modification (10, 18). A study of the effect of trypsin on the hemolytic activity of L Sendai is now in progress, and the preliminary experiments have clearly shown that trypsin treatment of L Sendai gained hemolytic activity to a level comparable to that of egg Sendai (*manuscript in preparation*).

Although the present data showed that the treatment of L Sendai with trypsin is followed by the activation of its infectivity for L cells, comparative studies between trypsin-treated and nontreated L Sendai on adsorption, penetration, and uncoating processes should be made to understand the trypsin-induced enhancement phenomenon in the present system.

ACKNOWLEDGMENTS

I would like to thank N. Ishida for interest and support, Y. Hinuma for criticism and R. O. Hatano for her supply of the fluorescent antibody.

Many of the data quoted were obtained by the generous cooperation of Takako Fukuda, for which I am greatly indebted.

This work was supported by a grant from the Ministry of Education, Japan.

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