Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 371-379

IMPROVED METHODS USING HVJ(Sendai virus) FOR INTRODUCING SUBSTANCES INTO CELLS

Tsuyoshi UCHIDA, Yasuko MIYAKE, Masaru YAMAIZUMI,
Eisuke MEKADA and Yoshio OKADA

The Research Institute for Microbial Diseases,
Osaka University, Yamada-kami, Suita, Osaka, Japan

Received January 29,1979

SUMMARY

When HVJ virions were sonicated in the presence of a given protein, about 0.2%-0.3% of total protein added was recovered in the virions. The protein molecules could be introduced effectively into cells. In this study, fragment A of diphtheria toxin was used as the test protein. More than 96% of L cells were killed after short exposure to the virus suspension containing fragment A diluted so as to contain only about 0.004 μg of fragment A per ml.

INTRODUCTION

HVJ(Sendai virus) is well known as a mediator of cell fusion(1). When the virus infects a susceptible cell, the viral envelope fuses with the cell and then the virion releases nucleoproteins into the cytoplasm of the cell. Reassembled HVJ envelopes containing non-toxic mutant protein(CRM45, about 45,000 daltons)(2) of diphtheria toxin with NAD:elongation factor 2-ADP ribosyl (NAD:EF2-ADPR) transferase activity(3,4) were prepared for introducing the protein effectively into all cells exposed to the vesicles. The protein was introduced into the cytoplasm as a result of fusion of the cells with the reassembled envelopes and thus became toxic to the cells(5). When any macromolecule is introduced into the HVJ virions by some means, it can then be intoduced effectively into susceptible mammalian cells by the fusion reaction of the virus. We report here an improved method using HVJ for introducing a given protein into cells. In this study, fragment A of diphtheria toxin was used as the test protein for two reasons. First, since fragment A (about 22,000 of

0006-291X/79/060371-09$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

molecular weight)(6,7) is a NAD:EF2-ADPR transferase(8,9) and thus inhibits protein synthesis in eukaryotic cell extracts, the amount of fragment A can be quantitatively determined by measuring residual enzymic activity. Second, fragment A in the culture medium is not toxic to sensitive cells because it has no fragment B to bind it to the cells(10) and thus cannot reach the cytoplasm, but when it is introduced into the cytoplasm it kills the cells by inhibiting protein synthesis(5,11,12).

HVJ is a kind of enveloped virus, the envelope being composed of lipids(13) and glycoproteins(14,15). Thus we expected that when the virus suspension was sonicated, the envelopes would be partially opened, and that proteins in the suspension would be trapped in the virions when the envelopes were closed again. In fact, fragment A molecules were readily trapped in the virions by sonication and then could be introduced into the cytoplasm of living cells. Thus virions containing fragment A blocked protein synthesis and killed the cells.

MATERIALS AND METHODS

HVJ(Sendai virus) and Fragment A of diphtheria toxin: HVJ(Sendai virus), Z strain, was propagated in the chorioallantoic of chick embryos. The virus was purified by differential centrifugation as described previously(16). Fragment A of diphtheria toxin was purified by DE52 and Sephadex G150 from culture supernatant of C7(β22) produced fragment A(17).

Preparation and purification of HVJ containing fragment A: A suspension of HVJ virions was mixed with fragment A. The mixture was sonicated in a Sonifier(Branson Ultrasonic Corporation model W185) at a dial 3 (23-25 Branson sonic power) setting at 0 C for 12 min (24 times for 30-second periods with a one-minute pause between sonications), and then layered on the top of 14 ml of 6% sucrose solution in phosphate buffered saline(PBS) in a centrifugation tube, and centrifuged at 27,000 rpm for 50 min. The resulting precipitate was resuspended in 1 ml of PBS and layered on 14 ml of a linear gradient of 10% to 30% sucrose in PBS and centrifuged at 25,000 rpm for 50 min at 4 C. Fractions of 1 ml were collected from the bottom of the tube, and aliquots of each fraction were used for determination of hemagglutinating activity and NAD:EF2-ADPR transferase activity. Before and after solubilization of the virions in each fraction with 0.5% NP40, the enzymic activity was determined using 5 μl, 10 μl and 20 μl samples by a slight modification of the method of Gill and Pappenheimer(8).

Determination of toxicity of HVJ containing fragment A to L cells: Suspensions of 300 L cells in 2 ml of MEM medium containing 10% calf serum were put into 30 mm plastic Petri dishes and incubated for 7.5 hour at 37C. Then the cells were washed once with 2 ml of chilled buffered salt solution (18) and 0.5 ml of chilled sample was added. After 20 min at 4 C the

dishes were warmed to 37 C and incubated for a further 25 min. Then 1 ml of culture medium was added and incubation was continued for 1 hour at 37 C. The medium was then replaced by fresh medium and the cells were cultured for 7 days. The cells were finally fixed with methanol and stained with Giemsa. Colony counts are averages of those in duplicate plates.

RESULTS AND DISCUSSION

1. Preparation and purification of HVJ virions containing fragment A of diphtheria toxin.

A mixture of purified fragment A and HVJ virions was sonicated for 12 min. Then the sonicated mixture was layered on the top of sucrose solution and centrifuged to separate the sonicated virions from free fragment A protein. A suspension of the resulting precipitate was subjected to sucrose gradient centrifugation. Sedimentation profiles showed that the peak fraction of HVJ virions in tube 4-6 was associated with both hemagglutinating activity and NAD:EF2-ADPR transferase activity (Fig. 1-A). Solubilization of the virions with Nonidet P40 (NP40) resulted in about 3.1-fold increase in enzymic activity, whereas there was no increase in the enzymic activity of the top fraction on treatment with NP40. These findings suggest that about 65%-70% of the total fragment A associated with the virions was entrapped inside the virions and about 35% - 30% was tightly bound to the virions. Control virions were mixed with fragment A protein at the same concentration and centrifuged under the same conditions without previous sonication. These control virions showed almost no enzymic activity and there was no further increase in activity after their solubilization with detergent. When virions were subjected to sucrose density gradient centrifugation analysis before and after sonication for 12 min, the sonicated virions were found in almost the same position as the control virions (Fig. 1-B). This observation suggests that HVJ virions were not broken into smaller particles by sonication. Cell fusion activity of the virus was not affected by sonication for 12 min. The hemagglutinating activity of the virus was increased about 2-fold by sonication for 4 to 12 min, but sonication for 16 min reduced the

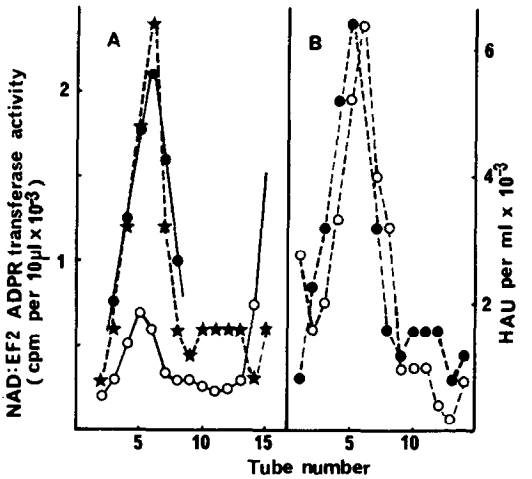

Fig. 1. Sedimentation analysis of HVJ virions sonicated in the presence of fragment A of diphtheria toxin.

(A) A suspension of HVJ virions was mixed with fragment A. The mixture was sonicated and then centrifuged as described in Materials and Methods. Hemagglutinating activity was expressed as HAU/ml(★). The NAD:EF2-ADPR transferase activities of each fraction before(—o—) and after (—●—) the treatment with NP40 are shown.
(B) A suspension of HVJ virions was devided into two portions, and one of the portions was sonicated as described above. Then each portion was subjected to sucrose density gradient centrifugation as described above and the HAU in each fraction was determined. (--o--)HAU before sonication, (--●--)HAU after sonication.

activity by half. Hemolytic activity of the virus was slightly increased by sonication for 12 min.

When 3-5 units of A540 nm ($3x10^4$-$6x10^4$HAU) of HVJ was suspended in 1 ml and sonicated for 12 min in the presence of fragment A, about 0.2 - 0.3% of the total fragment A added was recovered in the virions.

2. Toxicity of the HVJ virions containing fragment A to L cells.

Two kinds of virus suspensions (both about 5,000 HAU) were prepared containing fragment A equivalent in enzymic activity to about 0.13 µg/ml and 0.013 µg/ml of fragment A, and their toxicities were tested on mouse L cells. Table 1 shows that more than 96% of the L cells were killed after a short exposure to the virus suspension containing fragment A diluted so as to contain only 0.004 µg of

Table 1. Toxicity to L cells of HVJ virions containing fragment A.

Samples	HAU/ml	Number of surviving colonies per dish 5,000	1,700	500	170
Sonicated HVJ virions containing fragment A(0.13 μg)*		0	0	0	2
Sonicated HVJ virions containing fragment A(0.013 μg)**		1	8	28	63
Sonicated HVJ virions without fragment A		190	198	205	-
Native HVJ virions plus 1 μg fragment A***		-	218	211	-

Toxicity of each sample to L cells was determined as described in Materials and Methods. Control dishes contained 206 and 209 colonies. Samples were irradiated with ultra violet light before use.
* One ml of a suspension of sonicated virions(about 5,000 HAU/ml) containing fragment A was equivalent to about 0.13 μg of fragment A in enzymic activity.
** One ml of a suspension of sonicated virions(about 5,000 HAU/ml) containing fragment A was equivalent to about 0.013 μg of fragment A.
*** About 5,000 HAU of HVJ and 1 μg of fragment A were mixed in 1 ml.
- : not tested.

fragment A per ml (170 and 1,700 HAU). The same amount (HAU) of UV-killed virus mixed with 1 μg of free fragment A showed no toxicity at all on L cells. Control virions which were mixed with fragment A at the same concentration and washed by centrifugation under the same conditions without previous sonication showed also no toxicity on L cells when tested under the same conditions.

As described previously(5) when protein was enclosed within the reassembled HVJ envelopes, about 2% of the total protein added was recovered in the reassembled envelopes. With the present method only 0.2 - 0.3% of the total fragment A added was recovered in the virions, but a suspension of the virions containing only about 0.004 μg of fragment A was as toxic to L cells as reassembled envelopes containing 1.0 μg of CRM45(5). Thus, the sonicated virus is at least 20 times more effective for introducing proteins into the cytoplasm than the reassembled envelopes. Moreover, this method is simpler and easier than that using reassembled envelopes.

3. Methods for introduction of unstable substances when treated with prolonged sonication.

To shorten the sonication time, and thus reduce mechanical damage to unstable proteins we examined the effect of the length of sonication time on the amount of fragment A incorporated into the virions. For this, HVJ suspensions were sonicated in the presence or absence of fragment A for various times. In addition, virions sonicated in the absence of fragment A and the mixtures were sonicated for a further 2 or 4 min. Then the sonicated virions containing fragment A were passed through sucrose solution twice by centrifugation to remove free fragment A, and the amount of fragment A trapped in the virions was determined after solubilization of the virions with NP40. Results showed that when fragment A was added to suspensions of virions sonicated for 12, 10 or 8 min in the absence of fragment A, and these mixtures were then sonicated for a further 0, 2 and 4 min, the virions contained about 10%, 52% and 66%, respectively, of the fragment A trapped in virions sonicated in the presence of fragment A for 12 min(Fig. 2). Moreover, virions which were sonicated in the presence of fragment A for 8 min contained about 45% of the amount of fragment A which was trapped in virions sonicated for 12 min. Thus, when it is necessary to introduce a biologically or biochemically unstable substance that may be broken down during sonication for 12 min, a good procedure is to add it to virions that have been sonicated alone for 8 or 10 min and then sonicate the mixture for a further 4 or 2 min (see Fig. 2). In this way, about 0.1 - 0.2% of the substance can be introduced into the virions.

Another method using HVJ is also available for introducing an unstable substance into cells by reassembling the vesicles of HVJ envelopes with Bio-beads SM2 (Bio Rad Laboratories). For this, one ml of suspension of HVJ (about 10^5HAU) are solubilized with 1% Triton X100, and nucleocapsids and other insoluble materials are removed by ultra-

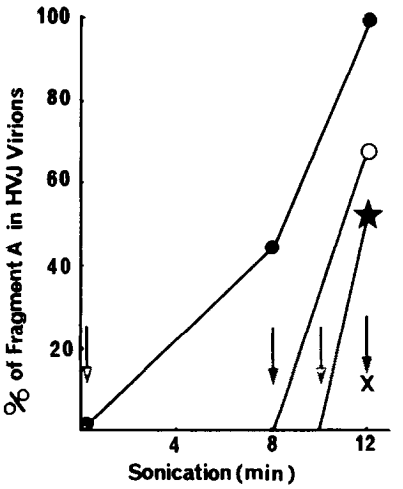

Fig. 2. Effect of the sonication time on incorporation of fragment A into HVJ virions.

Suspensions of HVJ virions(5.2 of A540, about 6 x 10^4HAU/ml) were sonicated in the presence of fragment A for 0, 8 and 12 min(●) under the conditions described in Materials and Methods and in the absence of fragment A. After sonication for 8, 10 and 12 min, 50μl of fragment A (9.4 mg/ml) was added to 1 ml of each sonicated suspension, and the mixtures were then sonicated for a further 4 (○), 2 (★) or 0 (×) min, respectively. The sonicated mixtures of virus and fragment A was centrifuged in the same way to remove free fragment A. NAD:EF2-ADPR transferase activity was assayed with 3 μl, 5 μl and 10 μl samples diluted 5-fold after NP40-treatment. The amount of fragment A was then calculated from the activity; 1.2 μg of fragment A was trapped in virus sonicated in the presence of fragment A for 12 min, and the enzymic activity of virus sonicated in the presence of fragment A for 12 min was taken as 100%. Arrow denotes addition of fragment A.

centrifugation. The test substance is then added to 1 ml of the solubilized envelope fraction and the mixture is slowly agitated for several hours in the presence of 1.0 g of Bio-beads SM2 that have been washed with methanol and distilled water(19). The resulting vesicles containing the substance are reassembled as the Triton is removed by the beads, and they are then separated from unreassembled envelope components and free substances by Bio-Gel A50m column chromatography and ultracentrifugation. These reassembled envelope-vesicles obtained using Bio-beads contain almost the same amount of fragment A as reassembled envelopes prepared by solubilization with NP40 and subsequent

dialysis. When the toxicity of the envelope-vesicles containing fragment A to L cells was determined as described in Materials and Methods, more than 90% of the L cells were killed after a short exposure to the vesicles diluted so as to contain about 0.03 μg of fragment A per ml.

These methods used HVJ should be useful for introducing various substances into the cytoplasm of living mammalian cells. When ^{125}I-bovine serum slbumin was introduced into the virions by this methods, the protein was recovered in the virions as effectively as fragment A.

There are many reports of methods for introducing substances into living mammalian cells: methods using liposomes(20, 21, 22) and erythrocyte ghost-fusion method (23, 24, 25) are frequently used. The HVJ-sonication method reported here is easy and efficient, because the resulting virions bind effectively to susceptible cells releasing their contents into cytoplasm.

REFERENCES

1. Okada, Y., and Tadokoro, J.(1962) Expl.Cell Res. 26: 108-118.
2. Uchida, T., Gill, D.M. and Pappenheimer, A.M.Jr.(1971) Nature New Biol. 233; 8-11.
3. Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I.(1968) J. Biol. Chem. 243:3553-3555.
4. Gill, D.M., Pappenheimer, A.M.Jr., Brown, R., and Kurnick, J.J. (1969) J. Exp. Med. 129:1-21.
5. Uchida, T. Yamaizumi, M., and Okada, Y.(1977) Nature. 266:839-840.
6. Gill, D.M., and Dinius, L.L.(1971) J.Biol. Chem. 246:1485-1491.
7. Collier, R.J., and Kandel, J.J.(1971) J. Biol. Chem. 246:1496-1503.
8. Gill, D.M., and Pappenheimer, A.M. Jr.(1971) J. Biol. Chem. 246: 1492-1495.
9. Drazin, R., Kandel, J., and Collier, R.J.(1971) J. Biol. Chem. 246: 1504-1510.
10. Uchida, T., Pappenheimer, A.M.Jr., and Harper, A.A.(1972) Science 175:901-903.
11. Yamaizumi, M., Uchida, T., Okada, Y., and Furusawa, M.(1978) Cell 13:227-232.
12. Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y. (1978) cell 15: 245-250.
13. Hosaka, Y., and Shimizu, K.Y.(1972) Virology 49:640-646.
14. Homma, M., and Ohuchi, M.(1973) J. Virol., 12:1457-1465.
15. Sheide, A., and Choppin, P.W.(1974) Virology 57:475-490.
16. Okada, Y. (1962) Expl. Cell Res. 26:98-107.
17. Uchida, T., Kim, J., Yamaizumi, M., Miyake, Y., and Okada, Y. in press J. Cell Biol.
18. Okada, Y., and Murayama, F.(1966) Expl. Cell Res. 44:527-551.
19. Holloway, P.W.,(1973) Anal. Biochem., 53:304-308.
20. Magee, W.E., and Miller, O.V.(1972) Nature, 235:339-340.

21. Gregoriadis, G., and Buckland, R.A. (1973) Nature, 244:170-172.
22. Papahadjopoulos, Poste, G., and Mayhew, E.(1974) Biochem. Biophys. Acta 363:404-418.
23. Furusawa, M., Nishimura, T., Yamaizumi, M., and Okada, Y.(1974) Nature, 249:449-450.
24. Loyter, A., Zakai, N., and Kulka, R.J.(1975) J. Cell Biol. 66:292-304.
25. Yamaizumi, M., Uchida, T., Okada, Y., Furusawa, M., and Mitsui, H. (1978) Nature, 273:782-784.