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Use of reconstituted Sendai virus envelopes for fusion-mediated microinjection of double-stranded RNA: inhibition of protein synthesis in interferon-treated cells

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Poly(I) · poly(C) molecules were trapped with reconstituted Sendai virus envelopes when added to the reconstitution system. A quantitative estimation indicated that about 10% of the added poly(I) · poly(C) remained associated with the fusogenic viral envelopes. About 50% of the associated poly(I) · poly(C) were found to be RNAase A resistant, enclosed within the viral envelopes. Incubation of loaded viral envelopes with HeLa or L-cells resulted in strong inhibition of protein synthesis, indicating fusion-mediated microinjection of the enclosed poly(I) · poly(C). Introduction of poly(I) · poly(C) into cultured cells by the use of reconstituted Sendai virus envelopes was as efficient as the introduction of these polynucleotides using the calcium phosphate coprecipitation technique. The inhibition of protein synthesis in L-cells but not in HeLa cells was dependent upon pretreatment with interferon. Incubation of poly(I) · poly(C)-loaded viral envelopes with interferon-treated variant cells of the NIH 3T3 line, which possess a very low amount of RNAase L, resulted in only 25% inhibition of protein synthesis, compared to 85% inhibition observed in L-cells

Introduction

Several proteins are induced in cell culture following interferon treatment Noteworthy among them are two enzymes which require double-stranded (ds) RNA as a cofactor for their activation (i) the (2′,5′)oligoadenylate synthetase [(2′-5′)A synthetase] which catalyzes the synthesis of oligonucleotides with the general structure $pppA(2'p5'A)_n$, these oligonucleotides bind to and activate a latent ribonuclease (RNAase L) [1,2], and (ii) a protein kinase which phosphorylates predominantly two cellular proteins, eIF_2 and p67, in interferon-treated cells [3,4] Some RNA viruses produce dsRNA during their replication, it is conceivable, therefore, that activation of the (2′-5′)A synthetase observed in viral-infected, interferon-treated cells is mediated by the viral dsRNA [5] Indeed, several lines of evidence have implicated inhibition of RNA viruses such as encephalomyocarditis and reo viruses in interferon-treated cells, with the activation of the (2′-5′)A synthetase-RNAase L pathway [5–7]

As a model system, synthetic dsRNA was introduced into interferon-treated cells, either by coprecipitation with calcium phosphate or with DEAE-dextran, in order to study the activation of the above enzymatic pathways independently of virus replication [8,9] Introduction of poly(I) poly(C) into interferon-treated cells was shown to activate the (2′-5′)A synthetase and to inhibit protein synthesis However, poly(I) poly(C) intro-

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Abbreviations poly(I) poly(C), polyinosinic-polycytidylic acid, PMSF, phenylmethylsulfonyl fluoride

duced into cells by the above technique did not block specifically virus replication but caused inhibition of cell protein synthesis

It is reasonable to assume that dsRNA introduced into cells during virus infection reaches a different intracellular compartment [5] than polynucleotides introduced via calcium phosphate [8] or DEAE-dextran techniques [9] In the present work, we have studied the possibility of using reconstituted Sendai virus envelopes (RSVE) as a vehicle to introduce poly(I) poly(C) into cultured cells It is likely that such a system would resemble the process of virus infection more than the introduction of poly(I) poly(C) via calcium phosphate or DEAE-dextran

Reconstituted Sendai virus envelopes are fusogenic vesicles consisting of the viral envelope phospholipids and their glycoproteins, but devoid of the viral genomic RNA [10] Fusion of loaded reconstituted Sendai virus envelopes with cells in culture leads to the introduction of the enclosed macromolecules into the recipient cell cytoplasm [11,12]

Materials and Methods

chemicals [^{3}H]Leucine was purchased from the Nuclear Research Center, Negev Israel Poly(I) poly(C) was obtained from Sigma Chemical Co Purified mouse interferon-α,β (1 10^8 international units/mg) was induced in L-cells, and purified on antibody affinity columns as previously described [13] Purified human interferon-α (3 6 10^6 units/mg) was a generous gift from Drs T Bino and H Rosenberg of the Biological Institute, Nes Ziona, Israel

Cells Human HeLa cells, mouse L929 cells and mouse NIH 3T3 cells clone 1 [14] were maintained in RPMI 1640 medium containing 10% calf serum

Introduction of poly(I) poly(C) into cultured cells by coprecipitation with calcium phosphate Poly(I) poly(C) molecules were introduced into cultured cells by the calcium phosphate coprecipitation technique, essentially as described before [8] Briefly, cells (1 10^5) were seeded in 16 mm wells with 0 5 ml RPMI 1640 medium containing 10% calf serum When needed, interferon was added 24 h after plating, and the cells were incubated for an additional 24 h Transfection buffer (20 mM Hepes buffer (pH 7 09), 136 mM NaCl, 5 mM KCl, 0 6 mM Na_2HPO_4, 10 mM NH_4Cl and 9 mM glucose), containing 5 μg poly(I) poly(C), was freshly prepared before each experiment, as described earlier [8] $CaCl_2$ was added to each dilution to a final concentration of 114 mM, and 0 25 ml of this suspension was applied to the cells After removal of the calcium phosphate suspension, growth medium was added for an additional 2 h of incubation, then the medium was removed and 0 5 ml of minimal essential medium (MEM), lacking leucine but containing 4μCi of [^{3}H]leucine, was added After 30 min at 37°C, the rate of protein synthesis was determined by the amount of [^{3}H]leucine incorporated into trichloroacetic acid-insoluble material [8] Maximum incorporation (100%) was the amount of [^{3}H]leucine incorporated into cells incubated in the absence of poly(I) poly(C)

Enclosure of poly(I) poly(C) in reconstituted Sendai virus envelopes Reconstituted Sendai virus envelopes were obtained after solubilization of intact Sendai virus particles by Triton X-100, essentially as described elsewhere [10,11] Briefly, a pellet of Sendai virus particles (1 5 mg protein) was dissolved in 30 μl of a solution containing 10% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 7 4) and 0 1 mM PMSF (Triton X-100 protein ratio, 2 1, w/w) To the clear supernatant obtained after centrifugation, poly(I) poly(C) dissolved in solution A (160 mM NaCl, 20 mM Tris-HCl, (pH 7 4)) was added to give a final concentration of 5–20 mg/ml and a final volume of 150 μl Triton X-100 was removed from the supernatant by direct addition of 40 mg of SM-2 Bio-Beads, as described before [12] The turbid suspension obtained (containing reconstituted Sendai virus envelopes) was centrifuged at 100 000 $\times g$ for 1 h The pellet, containing about 10% of the original viral protein, was then suspended in solution A to give a final protein concentration of 25 μg/ml

Fusion-mediated microinjection of poly(I) poly(C) into cultured cells A suspension of viral envelopes (5 μg/200 μl), loaded with 2 5 μg of poly(I) poly(C) (if not otherwise stated), was added to cultured cells (2 10^5 cells in 16 mm wells) The cultures were then incubated for 10 min at 4°C and 25 min at 37°C, after which the cells

were washed with MEM containing 5% serum, followed by 4 h of incubation at 37°C Medium was removed, cells were washed twice with MEM without amino acids, and MEM lacking leucine but containing 4 μCi/ml of [^{3}H]leucine was added Determination of protein synthesis was as described above

The concentration of poly(I) poly(C) (enclosed within or absorbed by the viral envelopes) was determined from optical absorption at 260 nm, after solubilization of the loaded viral envelopes with SDS (1%, w/v) As a standard, a solution of poly(I) poly(C) in 0 5 ml of solution A was used, with or without solubilized viral envelopes The contribution of the solubilized membrane proteins and phospholipids to the optical absorption of poly(I) poly(C) at 260 nm did not exceed 10% Protein concentration was determined according to the method of Lowry et al [15], using bovine serum albumin as a standard Each of the experiments described in the present work was repeated independently at least three times However, the results given in the tables and figures represent data obtained from a single specific experiment Quantitation differences between the various experiments never exceeded ± 5–7%

Results

When Sendai virus envelopes were reconstituted in the presence of poly(I) poly(C), about 10% of the added nucleotides were found to be associated with the viral vesicles (RSVE) formed after removal of the detergent (not shown) Approximately half of the viral vesicle-associated poly(I) poly(C) was removed by digestion with high levels of RNAase A Thus, about 5% of the total added nucleotides (300 μg poly(I) poly(C)/ 600 μg viral proteins) were trapped within the viral envelopes and protected from RNAase A digestion

Fig 1 shows that incubation of loaded, reconstituted envelopes with cultured HeLa cells causes strong inhibition of protein synthesis Empty vesicles, namely, envelopes which were reconstituted in the absence of poly(I) poly(C), did not inhibit protein synthesis unless added at concentrations higher than 5 μg/2 10^5 cells In subsequent experiments, 5 μg of reconstituted Sendai

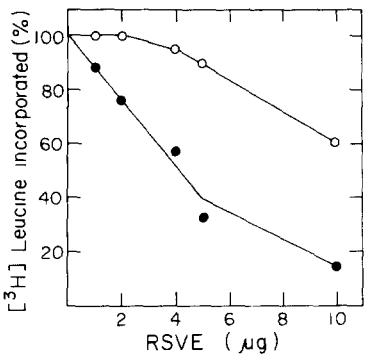

Fig 1 Inhibition of protein synthesis in HeLa cells by reconstituted Sendai virus envelopes (RSVE) loaded with poly(I) poly(C) Effect of increasing concentrations of viral envelopes Sendai virus envelopes were reconstituted in the presence (●) or absence (○) of poly(I) poly(C), and then incubated with HeLa cells as described in Materials and Methods Incorporation of [^{3}H]leucine in the absence of vesicles (100%) was 170000 cpm Although the data given represent the results obtained from a single experiment, essentially the same results were obtained from two additional experiments

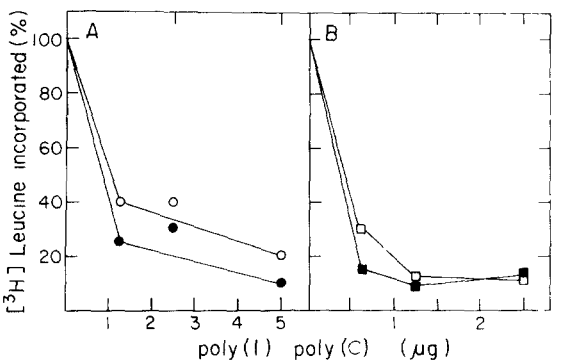

Fig 2 Inhibition of protein synthesis in HeLa cells after fusion mediated microinjection of poly(I) poly(C) into reconstituted Sendai virus envelopes or by poly(I) poly(C) coprecipitated with calcium phosphate (A) Reconstituted Sendai virus envelopes containing increasing amounts of poly(I) poly(C) were obtained by addition of increasing amounts of poly(I) poly(C) to a constant amount (900 μg) of detergent-solubilized viral glycoproteins All other conditions were as described in Materials and Methods Loaded reconstituted Sendai virus envelopes (5 μg), containing the indicated amounts of poly(I) poly(C), were incubated with HeLa cells untreated (○) or interferon treated (100 units/ml (●) (B) Increasing amounts of poly(I) poly(C) were coprecipitated with a constant amount of calcium phosphate and added to untreated (□) or interferon-treated (100 units/ml) (■) HeLa cells Extent of protein synthesis was determined after 4 h of incubation, as described in Materials and Methods Incorporation of [^{3}H]leucine in control cells (100%) was 70000 cpm

virus envelopes were used per 2 10^5 cells, a concentration at which cell protein synthesis was not significantly affected by empty vesicles (Fig 1)

The inhibition of protein synthesis was dependent upon the amount of poly(I) poly(C) entrapped within the viral envelopes (Fig 2A) Incubation of viral envelopes (5 μg) containing 1 25 μg of poly(I) poly(C) with HeLa cells, led to 70% inhibition of protein synthesis It seems that the efficiency of the reconstituted Sendai virus envelopes as a vehicle for introducing poly(I) poly(C) into HeLa cells is comparable to that of the complex calcium phosphate-poly(I) poly(C) (compare Fig 2A with 2B)

The results presented in Fig 2 also show that inhibition of protein synthesis in HeLa cells by poly(I) poly(C) was independent of interferon treatment When poly(I) poly(C) was introduced into a different cell line such as mouse L-cells, its effect was found to be interferon dependent in a dose-response manner (not shown) Similarly to the results with HeLa cells, those obtained with L-cells demonstrate that the two systems, i e , reconstituted Sendai virus envelopes and calcium-phosphate coprecipitation, are equally effective in the introduction of poly(I) poly(C)

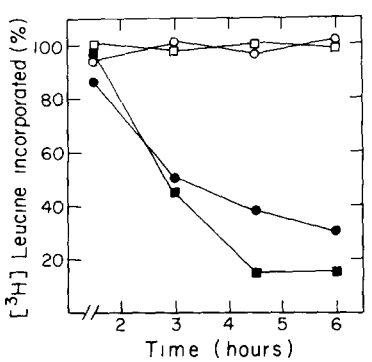

Fig 3 Inhibition of protein synthesis in L-cells treated with interferon Kinetic study Untreated (○, □) or interferon-treated (200 units/ml) (●, ■) were incubated with either calcium phosphate-poly(I) poly(C) complex (□, ■) or with reconstituted Sendai virus envelopes loaded with poly(I) poly(C) (○, ●), as described in Materials and Methods [^{3}H]Leucine incorporation was estimated at various times after the addition of the loaded reconstituted Sendai virus envelopes or the calcium phosphate-poly(I) poly(C) complexes Control plates treated with either calcium phosphate or viral envelopes without poly(I) poly(C), were assayed in parallel at each time point and taken as 100%

Kinetic studies showed that the inhibition of protein synthesis by poly(I) poly(C), introduced into L-cells by either of the two above methods, was progressive and reached about 50% inhibition after 3 h of incubation (Fig 3) It is noteworthy that at long incubation periods there was no re-

TABLE I

FUSION-MEDIATED INTRODUCTION OF poly(I) poly(C) INTO L-CELLS REQUIREMENT FOR ACTIVE FUSOGENIC VIRAL ENVELOPES

Sendai virus envelopes were loaded with poly(I) poly(C) and treated with trypsin, PMSF or dithiothreitol (DTT), as described in Materials and Methods and in a previous work [16] For digestion with RNAase A, loaded reconstituted Sendai virus envelopes (about 100 μg of viral protein) were suspended in 10 mM Tris-HCl buffer (pH 8 7) and then incubated with 6 μg RNAase A (Sigma Chemical Co), in a w/w ratio of 1 8 RNAase A poly(I) poly(C) After 60 min incubation at 30°C, the viral envelopes were collected by centrifugation and resuspended in Solution A to give 1 mg viral protein/ml Concentrations of poly(I) poly(C) were determined as described in Materials and Methods The loaded envelopes as well as poly(I) poly(C)-calcium phosphate complexes were incubated with L-cells pretreated with interferon (200 units/ml), as described in Materials and Methods Before coprecipitation with calcium phosphate, the poly(I) poly(C) was treated with RNAase A as follows 25 μg RNAase A were added to 200 μg of poly(I) poly(C) in 1 ml of 100 mM Tris-HCl (pH 8 7) The reaction mixture was incubated for 60 min at 30°C, at the end of which 25 mM EDTA were added and the mixture was then incubated for 5 min at 90°C After centrifugation (10000 × g, 5 min), the poly(I) poly(C) in the supernatant was coprecipitated with calcium phosphate, as described in Materials and Methods

System	Protein synthesis (%)
Reconstituted envelopes	100
Reconstituted envelopes + external poly(I) poly(C)	99
Loaded reconstituted envelopes	55
Loaded reconstituted envelopes treated with RNAase A	52
Loaded reconstituted envelopes, trypsin-treated	94
Loaded reconstituted envelopes, DTT-treated	92
Loaded reconstituted envelopes, PMSF-treated	88
Calcium phosphate	100
Calcium phosphate-poly(I) poly(C)	14
Calcium phosphate with RNAase A-treated poly(I) poly(C)	79

covery from the poly(I) poly(C) treatment, and by 24 h most of the treated cells in the culture were not viable In the absence of interferon treatment no inhibition of protein synthesis was observed in L-cells during 6 h of incubation (Fig 3)

To analyze whether introduction of poly(I) poly(C) via loaded reconstituted Sendai virus envelopes into cultured cells was mediated by a virus-cell fusion process, the reconstituted Sendai virus envelopes were pretreated with agents known to inhibit fusogenic activity The results in Table I show that loaded reconstituted Sendai virus envelopes treated with either trypsin or PMSF caused very little, if any, inhibition of protein synthesis in interferon-treated L-cells Trypsin and PMSF have been shown previously to specifically inhibit the fusogenic activity of sendai virus particles, without affecting their binding ability [16] In addition, loaded reconstituted Sendai virus envelopes which were reduced with dithiothreitol, lost their inhibitory effect in interferon-treated L-cells (Table I) Dithiothreitol-reduced reconstituted Sendai virus envelopes are unable to attach to cell plasma membranes due to inactivation of the viral binding protein (HN glycoprotein) [17]

The view that poly(I) poly(C) is enclosed within the reconstituted Sendai virus envelopes is further supported by the results presented in Table I While digestion of free poly(I) poly(C) with RNAase abolished its inhibitory effect after coprecipitation with calcium phosphate, RNAase A had no effect on the poly(I) poly(C) enclosed within the virus envelopes

The inhibition of protein synthesis which follows the introduction of poly(I) poly(C) into L-cells, could be attributed either to an activation of the (2′-5′)A synthetase-RNAase L pathway or to activation of the protein kinase To differentiate between these two possibilities, poly(I) poly(C) was introduced into the NIH 3T3 clone 1 cell line Proliferating NIH 3T3 clone 1 cells possess very little, if any, RNAase L activity [18,19], thus, if protein synthesis inhibition is mediated by the (2′-5′)A synthetase-RNAase L pathway, these cells are expected to be resistant to the inhibitory effect of poly(I) poly(C) From the results in Table II, it is evident that poly(I) poly(C) enclosed within reconstituted Sendai virus envelopes or coprecipitated with calcium phosphate had only a small inhibitory effect on protein synthesis in proliferating NIH 3T3 clone 1 cells As can be seen, the inhibitory effect of the loaded reconstituted Sendai virus envelopes on protein synthesis in NIH 3T3 clone 1 cells was much smaller than that observed with L-cells The low level (25%) of inhibition observed in NIH 3T3 clone 1 cells by loaded viral envelopes appears to be significant, since treatment of the viral envelopes with PMSF blocked this inhibition (data not shown)

TABLE II

THE EFFECT OF LOADED VIRUS ENVELOPES (RSVE) AND CALCIUM PHOSPHATE-poly(I) poly(C) COMPLEXES ON PROTEIN SYNTHESIS IN NIH 3T3 CLONE 1 CELLS

All experimental conditions were as described in Materials and Methods L-cells or NIH 3T3 cells were pretreated with 200 units/ml of interferon The incorporation of [^{3}H]leucine in control cells (100%) was 57000 cpm for NIH 3T3 clone 1 and 130000 cpm for L-cells

System	Protein synthesis (%)	
	NIH 3T3 clone 1	L-cells
RSVE	100	100
Loaded RSVE	75	14
Calcium phosphate	100	100
Calcium phosphate-poly(I) poly(C)	80	38

Discussion

The results presented in this work clearly demonstrate that reconstituted Sendai virus envelopes can be used as an efficient vehicle for the introduction of poly(I) poly(C) into cultured cells Poly(I) poly(C) was first added to a detergent solution of the viral envelope glycoproteins Removal of the detergent led to the formation of viral envelopes containing entrapped poly(I) poly(C) Incubation of loaded reconstituted Sendai virus envelopes with either L- or HeLa-cells, led to a strong degree of inhibition of protein synthesis, indicating that the entrapped poly(I) poly(C) was transferred into the above cells, probably by fusion of the viral envelopes with the recipient cell membranes Support for the view that a membrane-fusion step mediated the introduction of the poly(I) poly(C) was obtained from experiments showing that non-fusogenic loaded viral envelopes

obtained by treatment with trypsin, PMSF or dithiothreitol caused very little or no inhibition of protein synthesis

The observed inhibition of protein synthesis may be due to activation of the (2′-5′)A synthetase-RNAase L pathway and not to activation of the protein kinase In proliferating NIH 3T3 clone 1 cells, the basal level of RNAase L activity is very low, while dsRNA-dependent protein kinase activity is normal [18,20] When loaded reconstituted Sendai virus envelopes were incubated with these cells, significantly less inhibition of protein synthesis was observed Furthermore, the inhibition of protein synthesis was interferon-dependent in L-cells but not in HeLa cells The latter cells have been shown to possess high constitutive amounts of (2′-5′)A synthetase, and interferon-treatment induced synthesis of the enzyme only to a low extent [21] In L-cells, on the other hand, the constitutive levels of this enzyme are very low [21]

It should be emphasized, however, that more direct experiments are needed in order to claim that the effect of poly(I) poly(C) in the present work is due merely to activation of the (2′-5′)A-synthase-RNAase-L pathway The possibility that the decrease effectiveness of the poly(I) poly(C) (either trapped within reconstituted Sendai virus envelopes or coprecipitated with calcium phosphate) in NIH 313 clone 1 cells may be due to either a low extent of reconstituted Sendai virus envelopes-cell fusion or low degree of uptake of the poly(I) poly(C)-calcium phosphate complexes, cannot be excluded

From the results presented in this work, it appears that loaded viral envelopes are almost as efficient as the calcium-phosphate complexes for the introduction of poly(I) poly(C) into cultured L- and HeLa cells It should be emphasized that the viral envelopes transfer their content into the cell cytoplasm, using the same mechanism as intact, enveloped viruses such as Sendai virus use, for penetration [22] Therefore, introduction of poly(I) poly(C) via viral envelopes offers a better system for elucidating the detailed mechanism by which dsRNA inhibits protein synthesis in interferon-treated cells and, especially, its specific effect on inhibition of virus replication Moreover, calcium phosphate appears to be toxic to many cells, so that its use is limited to the few cell lines that show high endocytic activity [23] Since most of the cultured cells have receptors for Sendai virions and can be infected by them [24], it is expected that loaded reconstituted Sendai virus envelopes will be able to introduce poly(I) poly(C) into a wide spectrum of living cells

It is tempting to speculate that eventually viral envelopes will be used, similarly to phospholipid liposomes [25], as a vehicle for the introduction of macromolecules, especially of nucleic acids, into specific tissues of whole animals Viral envelopes, with some modifications, loaded with poly(I) poly(C) or other molecules, may be potential antiviral agents

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