

Cytotoxicity acquired by ribosome-inactivating proteins carried by reconstituted Sendai virus envelopes

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Association of the ribosome-inactivating proteins (RIPs): pokeweed antiviral protein (PAP), gelonin, *Momordica charantia* inhibitor (MCI), with reconstituted Sendai virus envelopes (RSVE) was obtained without detectable loss of activities either of RIPs or of viral envelope glycoproteins. RIPs are inactive towards intact cells, but, once encapsulated in RSVE, they become cytotoxic. The concentration of RSVE-associated PAP, which causes 50% inhibition of protein synthesis by Friend erythroleukemic cells, is 0.5 ng/ml. Substances capable to inhibit the viral activities block the acquired cytotoxicity of RIPs associated to RSVE.

Ribosome inactivating protein Sendai virus Toxin Microinjection Hybrid Fusion

1. INTRODUCTION

Intensive efforts, in many laboratories, are devoted to develop biological tools capable of eliminating selected populations of cells. Specific cytotoxic agents have been prepared by conjugating toxins (diphtheria toxin, ricin or abrin) to suitable carriers, such as antibodies (for review see [1]). To overcome the disadvantages of using whole toxins (mainly connected with lack of specificity), their active fragments (A-chains) have been used to construct conjugates [1]. The A-chains, when separated from the cell-binding B-chains, are non-toxic to intact cells, and still retain their capability of inactivating enzymically the elongation factor 2 (diphtheria toxin A-chain), or

eukaryotic ribosomes (ricin and abrin A-chains). However, the isolated A-chains have several drawbacks, such as instability or contamination by whole toxin; moreover, antibodies are often less efficient than B-chains in mediating the entry of A-chains into cells [2].

Aim of the present experiments was to produce a cytotoxic agent wherein the active moiety retained full activity, and as much as possible of it could be delivered into cells. As active moieties we used some ribosome-inactivating proteins (*Momordica charantia* inhibitor, gelonin and pokeweed antiviral protein) which are easily purified from seeds, and act as the A-chains of ricin and abrin (for review see [3]). Like the A-chains, RIPs do not bind to cells, have a low intrinsic toxicity, and can acquire toxicity upon conjugation to con A [3], to monoclonal anti-Thy 1.1 antibody [5], and to the Fab' fragment of IgG [6], or by inclusion into liposomes [7]. Liposomes have the great advantage of carrying a larger amount of unmodified protein; however, they are internalized into cells by endocytotic processes, and consequently most of the macromolecules they deliver are degraded [8].

Abbreviations: CHO, Chinese hamster ovary; DTA, diphtheria toxin A-chain; DTT, dithiothreitol; ID_{50} , concentration causing 50% inhibition; MCI, *Momordica charantia* inhibitor; PAP, pokeweed antiviral protein (prepared from seeds, PAP-S [15]); PMSF, *p*-methylsulphonylfluoride; RIP, ribosome-inactivating protein; RSVE, reconstituted Sendai virus envelope

To overcome this difficulty, we used reconstituted envelopes of Sendai virus, which had been employed successfully to transfer into cell-active macromolecules (for review see [9]), among which the A-chain of diphtheria toxin [10].

RSVEs, like Sendai virions, bind to cell plasma membranes containing sialic acid through the viral haemagglutinating-neuraminidase glycoprotein HN. Subsequently the viral glycoprotein F mediates the fusion between viral envelope and recipient cell plasma membrane, and thus the viral content is transferred to the host cells [11].

The association of RIPs with RSVEs was quantitatively considerable and at least comparable to that of DTA. RSVE-mediated entry of RIPs into cells was higher, by some 3 orders of magnitude, as compared with the liposomal system [7]. As a result, RSVE-associated RIPs were highly toxic to CHO, and even more to Friend's erythroleukemic cells.

2. MATERIALS AND METHODS

2.1. Materials

Sendai viruses were prepared, and their activity determined as reported in [12]. Diphtheria toxin A-chain, prepared from nicked purified toxin [13], was a generous gift of L. Montanaro (Istituto di Patologia Generale, Bologna). Gelonin [4], MCI [14] and PAP [15] were purified from the seeds of *Gelonium multiflorum*, *Momordica charantia* and *Phytolacca americana*, respectively. RIPs were freeze-dried and stored at -25°C . RIPs and DTA were radiolabelled by the iodogen procedure [16] using 18.5 MBq of carrier-free [^{125}I]iodide (Amersham International).

2.2. Preparation of reconstituted viral envelopes

Association of RIPs or DTA with RSVE was obtained by following the procedures in [17]. Briefly, 10 mg of viral protein was dissolved in 0.4 ml of 150 mM NaCl, 20 mM Tris-HCl, pH 7.4 (TBS), containing 2% Triton X-100. After 1 h at room temperature, the insoluble material was removed by centrifugation ($100000 \times g$, 1 h, 4°C) and 2.5 mg of RIP or DTA in 0.4 ml TBS were mixed with the solubilised viral envelopes. Triton X-100 was removed by dialysis for 72 h in Spectrapor 2 (Spectrum Medical Industries) against 1 g of Bio Bead SM2 (Bio-Rad Laboratories) in TBS. The

formed vesicles were isolated by centrifugation ($100000 \times g$, 1 h, 4°C).

2.3. Protein synthesis

2.3.1. Cell-free system

Cell-free protein synthesis was measured as in [14] with a rabbit reticulocyte lysate [18]; for details see table 1.

2.3.2. Cells

Chinese hamster ovary (CHO) cells and mouse Friend's 745 erythroblastoid cell lines were cultured at 37°C as monolayers, and suspensions in Falcon Tissue-culture ware in Eagle's medium supplemented with 10% (v/v) foetal calf serum.

The effect on cellular protein synthesis was measured after addition of RSVE containing RIPs or DTA both in monolayer cultures in Limbro 24-well tissue-culture plates (CHO cells), and in suspension cultures in 12×75 mm Falcon tubes (Friend's cells). Confluent cultures (CHO cells) or 2×10^6 cells/ml (Friend's cells) were rinsed with Hank's solution and were incubated at 37°C for 30 min with various concentrations of RSVE-RIPs or RSVE-DTA, and then were rinsed twice with Hank's solution. Cells were incubated in Eagle's medium containing 5% heat-inactivated foetal calf serum, which after 6 h (CHO cells) or 21 h (Friend's cells) was substituted by fresh medium without serum and containing $1-2 \mu\text{Ci/ml}$ of L-4,5- $[^3\text{H}]$ leucine (spec.act. 55 Ci/mmol, Amersham International). After 1 h at 37°C the radioactivity incorporated was measured as in [19].

3. RESULTS

RIPs were associated to RSVE with different yields (table 1). Association of inhibitory proteins with RSVE was estimated from the recovery of ^{125}I -labelled proteins, and from the inhibitory activity on cell-free protein synthesis. Both methods may be inaccurate, the first one owing to low counts, and the second owing to possible inactivation of the protein during the entrapping procedure. When RIPs were released from RSVE by treatment with 1% (v/v) Triton X-100, a 2-3-fold increase of their activity was observed in all cases (table 1). Attempts to separate previously

Table 1

Association of inhibitory proteins with reconstituted Sendai virus envelopes and their effect on protein synthesis

RIP	ID_{50} /mg of native	ID_{50} /mg of RSVE protein		Protein recovered (μ g/mg RSVE)	
		RIP	+ Triton X-100	From activity	From 125 I
PAP	900 900 ^a	10 500	23 500	26.0	40–60
MCI	606 300 ^a	375	1000	1.6	< 10
Gelonin	80 000 ^a	1400	3400	42.5	18
DTA	3600 ^b	22	30	8.2	< 10

^a From [3]^b Determined in the present work

Protein synthesis was evaluated in the lysate system as described in the text. Reaction mixtures (62.5 μ l) contained: 10 mM Tris-HCl buffer, pH 7.4; 100 mM ammonium acetate; 2 mM magnesium acetate; 1 mM ATP; 0.2 mM GTP; 15 mM phosphocreatine; 3 μ g of creatine kinase; 0.05 mM amino acids (minus leucine); 0.095 μ Ci L-[14 C]leucine; 25 μ l rabbit reticulocyte lysate. When DTA was tested, 1 mM NAD was added to the reaction mixture. The incubations were conducted at 28°C for 5 min. The reaction was stopped with 1 ml of 0.1 M KOH, and the radioactivity incorporated into protein was measured as in [15]

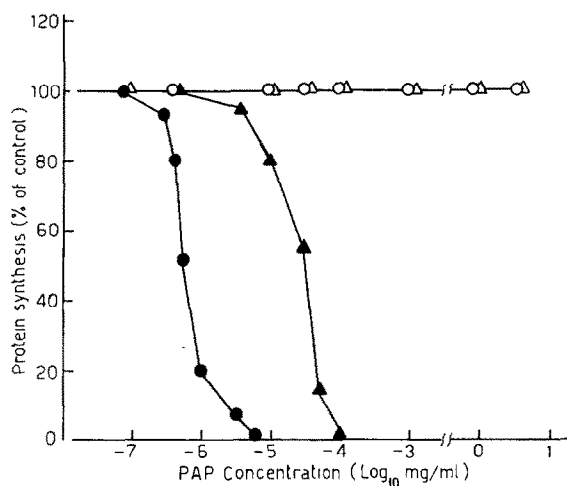


Fig.1. Inhibition of cellular protein synthesis by PAP associated with reconstituted Sendai virus envelopes. Cells were incubated for 30 min at 37°C, washed and then supplemented with MEM containing 5% inactivated foetal calf serum. Protein synthesis was measured after 21 h with Friend's cells and after 6 h with CHO cells as described in section 2. The amount of PAP associated with RSVE was estimated to be 50 μ g of PAP in 1 mg of reconstituted viral envelope proteins. (○---○) Friend's cells with PAP; (●---●) Friend's cells with RSVE-associated PAP; (▲---▲) CHO cells with PAP; (▲---▲) CHO with RSVE-associated PAP.

associated PAP from RSVE were unsuccessful.

RSVE-[125]PAP was incubated for 30 min at 37°C, either in 0.2 M phosphate buffer (pH 11), in 1 M NaCl, or in 2 M urea. The incubation mixtures were loaded on a sucrose gradient (40%, 20%, 10%, w/v), and were centrifuged at 100 000 $\times g$ for 1 h: a single peak of radioactivity was detected, which was associated with RSVE.

Preliminary experiments indicated that RSVE containing any of the RIPs used, or DTA, inhibited cellular protein synthesis, and that the respective ID_{50} 's were related to the amount of inhibitory protein associated with RSVE (not shown). Amongst the RIPs PAP was selected for further experiments, because it gave the highest activity associated with RSVE. Incubation with RSVE-PAP brought about a marked inhibition of protein synthesis by Friend's erythroleukemic cells, the ID_{50} being 0.5 ng/ml, comparable to that of ricin and abrin [20], whereas no inhibition of protein synthesis could be observed when the same cells were exposed to free PAP, at concentrations up to 1–2 mg/ml (fig.1). Similar results were obtained with RSVE-PAP and CHO cells, although in this case the ID_{50} was 15 ng/ml. This diversity could be due to the different incubation times after the 30 min exposure to RSVE-PAP (6 h with CHO and 21 h with Friend's cells). Addition to

Table 2

Cytotoxicity of PAP-associated RSVE to Friend's cells

Assay system	ng RSVE/ml ng PAP/ml	Protein synthesis (% of control)		
		1000 50	200 10	50 2.5
PAP alone		95	100	100
RSVE alone		95	95	100
PAP alone + empty RSVE		70	95	100
PAP-associated RSVE		—	—	5
PAP-associated RSVE pretreated with DTT ^a		—	90	100
PAP-associated RSVE pretreated with PMSF ^b		—	85	90

^a Treatment was performed by incubating 50 μ l of PAP-associated RSVE (viral protein 2.5 mg/ml) dissolved in medium A (pH 8.2) with 3 mM DTT at 37°C for 1 h. Then the mixture was diluted with Hank's solution before the assay

^b Treatment was performed by addition of 3 μ l of 210 mM PMSF (methanol solution freshly prepared) to 200 μ l of PAP-associated RSVE (viral protein 0.125 mg/ml). The mixtures were incubated for 30 min at 37°C, and then diluted in Hank's solution before the assay

Friend's cells were washed 3-times in Hank's solution, then the cells were exposed at 37°C for 30 min to the above system containing the indicated amount of PAP and/or RSVE

culture medium of mixtures of PAP and empty RSVE gave 30% inhibition of protein synthesis when 1 μ g of RSVE was added to cells in the presence of 50 ng of PAP.

To confirm that the internalization of RIPs was mediated by RSVE, viral glycoproteins were inactivated (table 2); in some experiments with a reducing agent (DTT) which caused the loss of binding activity, and consequently of fusion ability of RSVE, and in other experiments with PMSF, a specific inhibitor of virus-cell fusion [21]. RSVE-PAP treated with both reagents no longer inhibited protein synthesis of Friend's cells (table 2). The same reagents did not affect the inhibitory activity of PAP on cell-free protein synthesis.

4. DISCUSSION

A new cytotoxic agent, as potent as ricin and similar natural toxins, was obtained by associating ribosome-inactivating proteins (PAP, gelonin, MCI) with reconstituted Sendai virus envelopes. The RIPs used did not affect protein synthesis by intact cells, as indicated in [3] and confirmed by our results with CHO and Friend's cells. By contrast, as little as 0.5 ng/ml of RSVE-RIP is sufficient to cause 50% inhibition of protein synthesis in Friend's cells. Thus RIPs offer some advantages over DTA and probably the A-chains of other toxins in the preparation of cytotoxic agents with the use of RSVE. Our results suggest that this high toxicity acquired by RIPs is due to the ability of viral envelopes to fuse with plasma membrane, with concomitant delivery of the RSVE-RIPs to cells. This is confirmed by the low toxicity of RSVE-RIPs treated with PMSF, which inhibits the fusion, but not the binding of RSVE [21].

The experiments with RSVE-RIPs and RSVE-DTA disrupted with Triton X-100 suggest that as much as one half of inhibitory proteins remained exposed on the surface of the RSVE and apparently bound to viral envelope. Thus it is possible that the RSVE associated RIPs are transferred to the cells by a dual mechanism:

- (i) By delivery to the cytoplasm of the molecules entrapped inside the vesicles;
- (ii) By transfer to the host membrane of the molecules bound on the surface of the viral envelope [9].

RSVEs appear 1000-times more effective than liposomes (phosphatidylserine liposomes) in carrying RIPs [7]. As shown in [10], RSVEs are also effective in carrying DTA which was not delivered by liposomes [7]. This difference is likely to be due to the fusion ability of RSVE.

RSVEs bind to sialic acid residues on the cell surface, and consequently have a limited specificity for different cells. However, the fact that it is possible to conjugate an antibody to Sendai virus, after removal of unspecific binding activity [22], makes this viral envelope a candidate to target a large amount of fully active macromolecules towards specific target cells.

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