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Cellular Uptake and Protection against Virus Infection by Polyinosinic-Polycytidylic Acid Entrapped within Phospholipid Vesicles

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SUMMARY

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The high molecular weight, double-stranded RNA polyinosinic-polycytidylic acid ($rI_n \cdot rC_n$) can be entrapped by large (0.2–0.8-μm-diameter) unilamellar phospholipid vesicles. Increased cellular uptake of $rI_n \cdot rC_n$ (5–10-fold) was observed when human and mouse cells were treated with the polynucleotide entrapped within vesicles, compared with the uptake of free $rI_n \cdot rC_n$. Both rI_n and rC_n were taken up equally by cells in the entrapped form, whereas cells took up more rI_n than rC_n when treated with the free polynucleotide. Vesicle-entrapped $rI_n \cdot rC_n$ enhanced interferon induction and protection against vesicular stomatitis virus (5–10-fold) by cultured human cells, compared with free $rI_n \cdot rC_n$ at initially equal external concentrations. The results suggest that phospholipid vesicles can be used to potentiate the cellular uptake of nucleic acids in a functional form.

INTRODUCTION

Cultured cells *in vitro* can incorporate large numbers of phospholipid vesicles without cytotoxic effects (1–3). Entrapment of drugs and other substances within phospholipid vesicles considerably enhances their cellular uptake and biological effects (4–8). The characteristics of the uptake of vesicles by cells indicate that the most likely mechanism of incorporation is fusion with the plasma membrane of the cell (9–11), although certain types of vesicles can be incorporated primarily by endocytosis (11). Fusion of vesicles with the cellular plasma membrane provides the opportunity of introducing nonpermeable molecules directly into the cytoplasm of the recipient cells, and is thus a new experimental tool of obvious biological importance (3, 11, 12). Several pharmacological applications of this method have al-

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ready been reported both *in vitro* and *in vivo* (4, 6, 7, 13–18).

The development of a new technique for the formation of large unilamellar vesicles (19) has enabled us to extend the uses of lipid vesicles as carrier vehicles for the cellular incorporation of large macromolecules. Double-stranded synthetic polynucleotides such as $rI_n \cdot rC_n$[1] are of interest, as they induce interferon and have tumor-inhibitory effects (20). We report that (a) phospholipid vesicles can be used to stimulate $rI_n \cdot rC_n$ uptake by mouse and human cultured cells; (b) when $rI_n \cdot rC_n$ is entrapped in phospholipid vesicles, the components of both strands are taken up by cells equally; and (c) vesicle-entrapped $rI_n \cdot rC_n$ can increase interferon induction and viral protection in cultured human cells. These experiments demonstrate for the first time the potential usefulness of phospholipid vesicles to entrap and enhance the cellular uptake of nucleic acids in a functional form.

MATERIALS AND METHODS

Polynucleotides. rI_n, rC_n, $rI_n \cdot rC_n$, and [^{3}H]rC_n were obtained from Miles Laboratories. [^{14}C]rI_n and [^{3}H]rI_n were obtained from Schwarz-Mann. rI_n and rC_n, and [^{14}C]rI_n and [^{3}H]rC_n, were annealed to give the double-stranded labeled [^{14}C]$rI_n \cdot$ [^{3}H]-rC_n. The complex was characterized spectrophotometrically and had a specific activity of 0.7–1.6 $\times$ 10^5 dpm/10 μg. $rI_n \cdot$ [^{3}H]rC_n was made by annealing rI_n with [^{3}H]rC_n and had a specific activity of 1–2 $\times$ 10^5 dpm/10 μg.

Phospholipid vesicle preparation. Phospholipid vesicles were prepared as follows. Phosphatidylserine, chromatographically pure, was prepared as before (21), washed with EDTA, and stored in chloroform at low temperature under nitrogen (22). Ten micromoles of phosphatidylserine were suspended in 1 ml of 100 mM NaCl buffer at pH 7.4 and sonicated in a bath-type sonicator for 0.5 hr at 24° as described elsewhere (23). The NaCl buffer also contained 2 mM histidine, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 0.1 mM EDTA. To the optically clear sonicated suspension, Ca^{2+} was added (70 μl of 0.1 M $CaCl_2$), and the mixture was incubated for 1 hr at 37°. The white precipitate which developed soon after the addition of Ca^{2+} was then centrifuged at 10,000 $\times$ *g* for 10 min. The supernatant was removed.

Entrapment of $rI_n \cdot rC_n$. The wet pellet of phospholipid vesicles was mixed with 0.1 ml of a solution of either $rI_n \cdot rC_n$ or rI_n and rC_n (20 mg/ml) (Miles Laboratories) which had been dialyzed against 100 mM NaCl buffer, pH 7.4. The mixture was shaken with a few glass beads for 5 min at 37°, and then 80 μl of 0.1 M EDTA and 50 μl of 0.1 M NaOH were added. At this point the suspension was only slightly opalescent. It was shaken for 10 min at 37° and finally left to equilibrate for 30 min at the same temperature. The suspension was then diluted with 0.5 ml of 100 mM NaCl buffer and centrifuged at 48,000 $\times$ *g* for 20 min at 20°. The pellet, which contained most of the phospholipid, was resuspended in 5 ml of NaCl buffer and centrifuged as above; the process was repeated three times, and the final pellet was suspended in 1 ml of PBS free of Ca^{2+} and Mg^{2+}.

Virus preparation. Bovine vesicular stomatitis virus (New Jersey serotype) was harvested from infected mouse L-cells to yield a titer of 2 $\times$ 10^8 plaque-forming units/ml. The virus stock was diluted 20-fold and was stored at −70°.

Cellular uptake experiments. Mouse L929 cells were grown in medium 1640 containing 10% calf serum. Human neonatal foreskin fibroblasts (passages 5–24) were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cultures were seeded with 5 $\times$ 10^4–1 $\times$ 10^5 cells in 2 ml and were grown with rotation at 12 rph to approximately 1 $\times$ 10^6 cells/tube (approximately 50% confluency). On the day of the experiment the medium was decanted, and 2 ml of Dulbecco's PBS were added. Phospholipid vesicles prepared as described above were added to give a final concentration of $rI_n \cdot rC_n$ of 10 μg/ml, and of lipid phospho-

[1] The abbreviations used are: $rI_n \cdot rC_n$, polyinosinic-polycytidylic acid; PBS, Dulbecco's phosphate-buffered saline (NaCl, 0.137 M; $NaHPO_4$, 8.2 $\times$ 10^{-3} M; KCl, 2.7 $\times$ 10^{-3} M; KH_2PO_4, 1.9 $\times$ 10^{-3} M; $MgCl_2$, 1.1 $\times$ 10^{-3} M; $CaCl_2$, 0.9 $\times$ 10^{-3} M); TCA, trichloracetic acid.

rus, approximately 1 μmole/ml. The tubes were incubated for 1 hr, the supernatant medium was decanted off, and 5 ml of PBS were added; the mixture was centrifuged at 250 × *g* for 1 min and washed three times in 5 ml of PBS. The cells were lysed in 0.5 ml of water, and radioactivity was measured. Controls included tubes with no cells to determine binding of $rI_n \cdot rC_n$ and vesicles to glass surfaces. In initial experiments, TCA precipitation was used to determine whether the cellular radioactivity was associated with high molecular weight material. It was found that during the short-term incubation procedures reported here essentially all cell-associated radioactivity was TCA-precipitable, indicating that no significant degradation of $rI_n \cdot rC_n$ had occurred. Appropriate controls were made for ^{14}C-^{3}H double label counts. All readings were made in quadruplicate.

Antiviral activities. Human neonatal foreskin fibroblasts (passages 5–24), in monolayers, were exposed to the polynucleotide-vesicle complexes in PBS for 1 hr, washed, and reincubated for 18 hr in Eagle's minimal essential medium containing 2% fetal calf serum. Colorimetric assays for intracellular resistance to viral challenge (vesicular stomatitis virus, multiplicity of infection = 0.15) and for extracellular interferon titers were carried out as previously described (24, 25). Percentage of protection was defined as the ratio of dye uptake by viable cells in the infected culture to dye uptake by viable cells in a mock-infected culture.

RESULTS

Capture of polynucleotides by the vesicles was assayed radioisotopically and varied from 5% to 10% in different experiments. Figure 1 shows the appearance of such vesicles by freeze-fracture electron microscopy under conditions described earlier (19). They appear as spheroidal structures with diameters varying from 0.2 to 0.8 μm. Where the fracture has passed through the interior of the vesicle, one can observe smaller, entirely contained vesicles, usually with large aqueous spaces in between. Although they are clearly a multicompartment system, they are different from the multilamellar vesicles formed during the initial hydration and shaking of phosphatidylserine in aqueous salt solution. These latter structures, originally described by Bangham *et al.* (26), are larger, with more concentric lamellae separated by considerably smaller aqueous spaces.

Table 1 shows results of experiments in which mouse L929 cells and human fibroblasts (both in monolayer cultures) were incubated for 1 hr at 37° with $[^{14}C]rI_n \cdot [^{3}H]rC_n$, either in solution or entrapped within lipid vesicles. The radioactivity associated with the cells was found to be considerably higher (2.5–9-fold) when they were incubated with vesicle-entrapped $rI_n \cdot rC_n$ than with free $rI_n \cdot rC_n$ at the same concentration. In addition, the results indicate that vesicle entrapment stimulates the uptake of the rC_n strand more than the rI_n strand (6–9-fold vs. 3-fold). However, the use of the double-labeled $rI_n \cdot rC_n$ clearly shows that the original ^{3}H:^{14}C ratio in the starting material was essentially unaltered when vesicle-entrapped $rI_n \cdot rC_n$ was taken up by the cells. Thus we conclude that when free $rI_n \cdot rC_n$ is incubated with cells there is relatively less incorporation of rC_n, resulting in a lower ^{3}H:^{14}C ratio. This is consistent with a previous report that there is a differential cellular uptake of the two homopolymers even though they are initially present as a double-stranded structure (27). This result may be due to the greater susceptibility of rC_n to degradation by surface enzymes such as phosphodiesterases (27). In any case, our present results establish that vesicle entrapment prevents such effects and delivers apparently intact macromolecular $rI_n \cdot rC_n$ to cells, thus maintaining the initial rI_n:rC_n ratio. It is probable that other natural and synthetic polynucleotides, either single- or double-stranded, may be protected in a similar way, thus increasing the possibility of uptake by cells in a functional state. For example, the vesicle vehicle would be particularly useful for monitoring effects on cell functions of double-stranded RNAs with unusual biolability, such as mismatched interferon inducers (28).

Table 2 compares the uptake of free and vesicle-entrapped $rI_n \cdot rC_n$ and the single-stranded rI_n and rC_n by human fibroblasts.

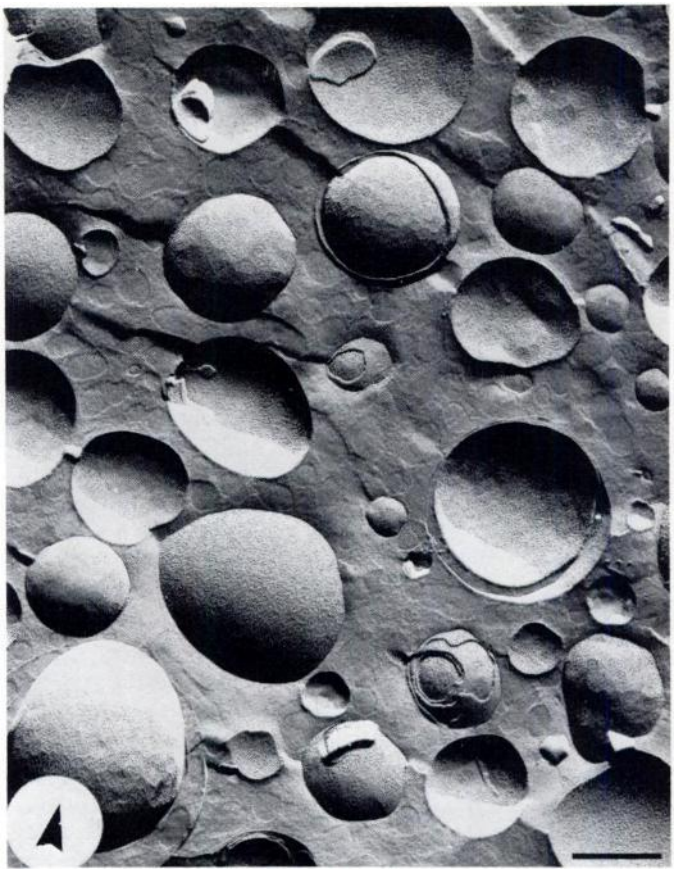

FIG. 1. *Large unilamellar vesicles (containing $rI_n \cdot rC_n$) as seen by freeze-fracture electron microscopy (19)* Horizontal bar = 0.3 μm. Arrow shows direction of shadowing. Vesicles were prepared as described in the text.

The uptake of all polynucleotides was significantly increased by vesicle entrapment. However, the stimulation of uptake of rC_n was greater than that of rI_n, supporting the results shown in Table 1.

Table 3 shows that incubation of human fibroblasts with vesicle-entrapped $rI_n \cdot rC_n$ produced a considerable increase in antiviral activity and interferon induction, compared with the effects of free $rI_n \cdot rC_n$ under the same conditions. Although the degree of stimulation varied somewhat among three experiments, the effect of vesicle entrapment can be seen clearly at several concentrations. The increased activity is of the same order (5–10-fold) as the increased uptake of polynucleotide shown in Table 1. Thus these effects of vesicle-$rI_n \cdot rC_n$ can be interpreted simply as the result of stimulated cellular $rI_n \cdot rC_n$ uptake. Incubation of human fibroblasts with free or vesicle-entrapped rI_n or rC_n did not elicit any detectable antiviral activity or interferon induction, indicating that the double-stranded $rI_n \cdot rC_n$ is necessary for the antiviral effects of both free and vesicle-entrapped polynucleotides. Vesicles alone had no activity at the highest concentration tested, and mixing of free $rI_n \cdot rC_n$ with previously formed lipid vesicles did not increase its activity. In other experiments, it was found that, at an initial external concentration up to 10 μg/ml, neither free nor vesicle-entrapped $rI_n \cdot rC_n$ inhibited the growth of human fibroblasts over a 2-day period. Also, there was no significant differential effect between free and entrapped $rI_n \cdot rC_n$ on incorporation of [^{14}C]amino acids into high molecular weight (TCA-precipitable) material by human fibroblasts, although they both inhibited incorporation slightly when com-

TABLE 1

Uptake of free and vesicle-entrapped [^{14}C]$rI_n \cdot$[^{3}H]rC_n by L929 cells and human fibroblasts

Mouse L929 cells were grown in medium 1640 containing 10% calf serum, and human neonatal foreskin fibroblasts (passages 5–24) were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cultures were seeded with 5×10^4–1×10^5 cells in 2 ml and were grown with rotation at 12 rph to approximately 1×10^6 cells/tube (approximately 50% confluency). On the day of the experiment the medium was decanted, and 2 ml of Dulbecco's phosphate buffered saline were added. Phospholipid vesicles prepared as described above were added to give a final concentration of $rI_n \cdot rC_n$ of 10 μg/ml, and of lipid phosphorus, approximately 1 μmole/ml. The tubes were incubated for 1 hr, the supernatant medium was decanted off, and 5 ml of PBS were added; the mixture was centrifuged at $250 \times g$ for 1 min and washed three times in 5 ml of PBS. The cells were lysed in 0.5 ml of water, and radioactivity was measured. Controls included the following. (a) Tubes were incubated without cells to determine binding of $rI_n \cdot rC_n$ and vesicles to glass surfaces. (b) In initial experiments TCA precipitation was used to determine whether the cellular radioactivity was associated with high molecular weight material. It was found that during the short-term incubation procedures reported here essentially all cell-associated radioactivity was TCA-precipitable, indicating that no significant degradation of $rI_n \cdot rC_n$ had occurred. (c) Appropriate controls were made for ^{14}C-^{3}H double label counts. (d) All readings were made in quadruplicate. (e) rI_n, rC_n, and [^{3}H]rC_n were obtained from Miles Laboratories; [^{14}C]rI_n was obtained from Schwarz-Mann. rI_n and rC_n, and [^{14}C]rI_n and [^{3}H]rC_n, were annealed to give the double-stranded labeled [^{14}C]$rI_n \cdot$[^{3}H]rC_n. The complex was characterized spectrophotometrically and had a specific activity of 0.7–1.6×10^5 dpm/10 μg. The specifications available from the suppliers indicated that the various polynucleotides used had a molecular weight of more than 2×10^5 (usually $s_{20} = 9$ S).

State of $rI_n \cdot rC_n$	[^{14}C]rI_n uptake/10^6 cells		[^{3}H]rC_n uptake/10^6 cells		^{3}H:^{14}C
	dpm	*% total*	*dpm*	*% total*	*dpm/dpm*
Experiment 1 (L929)					
Cell uptake (free)	371	1.04	620	0.42	1.67
Cell uptake (vesicles)	1,382	3.82	5,597	3.74	4.05
Initial (free), 10 μg/ml	35,700		147,630		4.13
Initial (entrapped in vesicles), 10 μg/ml	36,200		149,510		4.13
Stimulation of uptake, vesicles/free		3.67		8.90	
Experiment 2 (L929)					
Cell uptake (free)	244	0.59	342	0.29	1.40
Cell uptake (vesicles)	896	2.03	2,554	1.91	2.85
Initial (free), 10 μg/ml	41,220		119,540		2.90
Initial (entrapped in vesicles), 10 μg/ml	44,200		133,930		3.03
Stimuation of uptake, vesicles/free		2.91		6.59	
Experiment 3 (fibroblasts)					
Cell uptake (free)	184	0.46	167	0.19	0.91
Cell uptake (vesicles)	481	1.23	988	1.07	2.05
Initial (free), 10 μg/ml	40,090		88,120		2.20
Initial (entrappd in vesicles), 10 μg/ml	38,980		92,370		2.37
Stimulation of uptake, vesicles/free		2.67		5.63	

pared with controls. Presumably these effects are due to the known inhibitory effects of nanogram levels of double-stranded RNA on the initiation of protein biosynthesis (29). These results indicate that vesicle-entrapped $rI_n \cdot rC_n$ is not grossly cytotoxic to human fibroblasts, and that at comparable extracellular concentrations it is a much stronger inducer of interferon compared with free $rI_n \cdot rC_n$.

DISCUSSION

This report is the first demonstration of enhanced interferon induction *in vitro* by

TABLE 2

Uptake of free and vesicle-entrapped $rI_n \cdot rC_n$, rI_n, and rC_n by human fibroblasts

These experiments, using human fibroblasts, were carried out as described in the legend to Table 1, except that $rI_n \cdot [^3H]rC_n$, $[^3H]rI_n$, and $[^3H]rC_n$ were used. $rI_n \cdot rC_n$, rI_n, rC_n, and $[^3H]rC_n$ were obtained from Miles Laboratories; $[^3H]rI_n$ was obtained from Schwarz-Mann. $rI_n \cdot [^3H]rC_n$ was made by annealing rI_n with $[^3H]rC_n$. The specific activities of the polynucleotides varied from 1 to 2×10^5 dpm/10 μg. The initial external concentration of the polynucleotides, free or vesicle-entrapped, was 10 μg/ml, and the data from two separate experiments are expressed as the percentage uptake (from radioactivity determinations) per 10^6 cells.

State of polynucleotide	$rI_n \cdot [^3H]rC_n$		$[^3H]rI_n$		$[^3H]rC_n$	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	% uptake		*% uptake*		*% uptake*	
Free	0.26	0.31	0.38	0.44	0.16	0.23
Vesicle-entrapped	1.22	1.68	1.01	1.12	0.91	1.00
Stimulation of uptake	4.69	5.42	2.66	2.32	5.68	4.35

TABLE 3

Interferon induction and protection of human fibroblasts against vesicular stomatitis virus by free and vesicle-entrapped $rI_n \cdot rC_n$

Human neonatal foreskin fibrolasts (passages 5–24) were grown as monolayers as described in legend to Table 1. The cells were exposed to the polynucleotide-vesicle complex in PBS for 1 hr, washed, and reincubated in fresh medium at 37° for 18 hr. To determine intracellular viral protection, vesicular stomatitis virus (VSV) was used as the challenge virus. Colorimetric assays, using neutral red for intracellular resistance to viral challenge and for extracellular interferon titers, were carried out as described previously (28). $rI_n \cdot rC_n$, rI_n, and rC_n were obtained from Miles Laboratories. Tracer quantities of $rI_n \cdot [^3H]rC_n$, $[^3H]rI_n$, and $[^3H]rC_n$ were used to determine amounts of polynucleotides trapped in vesicles. The amount of lipid present was approximately 1 μmole/10 μg of polynucleotide.

Inducer	Initial external concentration	Protection against VSV			Interferon titer	
		Expt. 1	Expt. 2	Expt. 3	Expt. 2	Expt. 3
	μg/ml	%	%	%		
$rI_n \cdot rC_n$	0.1			0		<1
	1.0	12	36	4	1	<1
	10.0	83	46	43	2	5
rI_n	1.0			0		<1
	10.0			0		<1
rC_n	1.0			0		<1
	10.0			0		<1
Vesicle-entrapped	0.1			9		<1
$rI_n \cdot rC_n$	1.0	100	42	67	6	3
	10.0	100	100	94	16	19
Vesicle-entrapped	1.0			0		<1
rI_n	10.0			0		<1
Vesicle-entrapped	1.0			0		<1
rC_n	10.0			0		<1
Vesicles only	~1 μmole/ml	0	0	0	<1	<1

$rI_n \cdot rC_n$ entrapped within large unilamellar lipid vesicles. Recently it was noted that $rI_n \cdot rC_n$ entrapped in "conventional" phospholipid vesicles (liposomes) did not significantly stimulate interferon induction in L929 cells *in vitro*, although there were considerably increased effects *in vivo* in mice (18). The lack of effects in that system *in vitro* might be due to one or more of the following factors: (a) the L929 cells, which, unlike human cells, require prior threatment with a cation for interferon induction, were refractory to $rI_n \cdot rC_n$ alone; (b) the lipid vesicles used were also of the multilamellar type and were found to be cytotoxic in contrast to the results in our system; (c) in the above report (18) the polynucleotides were probably "bound" to

the surface of the lipid bilayers by electrostatic interactions, since most of these vesicles were positively charged. In support of all three notions, initial experiments in our laboratory with $rI_n \cdot rC_n$ "bound" to positively charged vesicles did in fact indicate a decrease in ability to induce interferon compared to free $rI_n \cdot rC_n$, although its uptake by the cells was considerably enhanced. Effect of $rI_n \cdot rC_n$ with different types of lipid vesicles are now being studied in our laboratory.

One of the interesting aspects of interferon induction by $rI_n \cdot rC_n$ is the still unanswered question whether the inducer must enter target cells. Evidence from the effects of polynucleotides bound to immobilized surfaces supports the notion that induction is mediated exclusively by the cell surface. However, this has not been proven unequivocally (24, 28, 29), primarily because of persistent small leakage of $rI_n \cdot rC_n$ bound to the immobilized surface (30–32). In this communication we emphasize that our measurements of cell-associated radioactivity do not give any direct information on the cellular site of binding. However, a detailed examination of the properties of our system reveals pertinent insights concerning the possible site of action for $rI_n \cdot rC_n$ in inducing interferon.

1. Since both lipid vesicles and the polynucleotides are strongly and uniformly negatively charged, it is unlikely that the $rI_n \cdot rC_n$ is "bound" on the surface of the vesicle membrane. (In fact, the amount of $rI_n \cdot rC_n$ found to be associated with the lipid vesicles is in reasonable correspondence with the amount of passively captured sucrose, indicating entrapment within the interior aqueous space of the vesicle.) Therefore it is reasonable to conclude that the stimulation of interferon production by entrapment within vesicles as reported here is due to enhanced cellular uptake of $rI_n \cdot rC_n$ rather than "surface activation."

2. Incorporation of negatively charged fluid vesicles composed of material similar to that used here has been shown to involve predominantly fusion with the cellular plasma membrane (11). It appears reasonable, then, to expect that following such a fusion event, the polynucleotide initially entrapped within the lipid vesicle will be released directly into the cytoplasm, bypassing any hypothetical "receptor" on the cell surface. Although the above considerations do not disprove a physiological role for such receptors at the cell surface, they do support the notion that $rI_n \cdot rC_n$ can be active in inducing interferon when introduced intracellularly. A more rigorous, long-term approach to the question of the location of the interferon inducer receptor would include experiments in which entrapped $rI_n \cdot rC_n$ is added to cells already maximally stimulated with free $rI_n \cdot rC_n$. Such experiments would help to determine whether there are both membrane and cytoplasmic receptors.

It is obvious from the above arguments and the results reported here that phospholipid vesicles hold substantial promise as a new experimental tool in cell biology. By fusion with the plasma membrane they provide the opportunity of introducing directly into the cytoplasm large macromolecules in an active form. They can thus bypass both the problem of lysosomal digestion, which normally follows endocytosis, and the need for special receptors at the cell surface, as, for example, with various classes of animal viruses. In fact, the role of such receptors can be examined in detail with this method. Since both the entrapment of macromolecules within the vesicles and the fusion of vesicles with cells are relatively nonspecific, this method can be applied to various large macromolecular assemblies, including whole viruses. Such studies are now in progress.

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