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Antibody targeted liposomes containing poly(rI) · poly(rC) exert a specific antiviral and toxic effect on cells primed with interferons α/β or γ

Pierre G. Milhaud [1], Patrick Machy [2], Bernard Lebleu [1] and Lee Leserman [2]

[1] *Laboratoire de Biochimie des Protéines, URA CNRS 1191, Université des Sciences et Techniques du Languedoc, Montpellier and* [2] *Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Marseille (France)*

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Double-stranded RNA can stimulate interferon production and mediate an antiproliferative effect on certain cell types. We evaluated the possibility of specifically targeting to cells in vitro the RNA duplex poly(rI) · poly(rC) in pharmacologically active form after its encapsulation in small, unilamellar liposomes, to which was covalently coupled protein A. These liposomes became bound to and were endocytosed by murine L929 cells in the presence of protein A-binding monoclonal antibodies specific for an expressed cell surface protein, the H-2K molecule. When L929 cells were preincubated in the presence of low doses of interferon α/β or γ, they could be activated to produce interferon following exposure to either free poly(rI) · poly(rC), or specifically bound liposome poly(rI) · poly(rC), but not the same liposomes in the presence of non-cell binding control antibodies. Specifically bound liposome-encapsulated poly(rI) · poly(rC) was toxic to L929 cells at dose levels at least three logs lower than free poly(rI) · poly(rC). This toxicity was also dependent on pre-treatment with interferon. These results indicate that liposome-encapsulated poly(rI) · poly(rC) can survive endocytosis and can be released in active form to specific cell populations, at concentrations much lower than that required for pharmacologic effects of the same molecule in free form. They suggest that introduction into cells of other nucleic acids might benefit from the antibody-targeted liposome technology described here.

Introduction

For reasons of interest both in basic science and for possible therapeutic applications it would be useful to be able to introduce nucleic acids into cells in a reproducible and efficient manner. In our laboratories we have studied antiviral oligo- and poly-nucleotides such as (2′-5′)$(A)_n$, a mediator of interferon (IFN) action [1], the synthetic double-stranded RNA (dsRNA) duplex poly(rI) · poly(rC) (poly IC), which acts as an IFN inducer [2], and anti-sense oligonucleotides [3]. Poly IC and IFN act synergistically to stimulate the production of IFN and to induce an antiproliferative effect on certain cell lines [4,5]. Poly IC is also an inducer of several genes [6,7]. Experiments with viral mutants indicated that a single molecule of dsRNA, introduced intracellularly via replication-defective viral strains, was sufficient to induce the production of IFN [8]. This has been confirmed by direct microinjection of poly IC into the cytoplasm [9,10]. Oligo- and poly-nucleotides are also known to be taken up by cells in culture, but this process is inefficient: when dsRNA was presented in medium outside the cells at least 10^5 RNA molecules were necessary for IFN induction. This may be due to the degradation of RNA by nucleases, its inefficient entry into cells, or both [8,11]. Poly IC thus appears to be a useful model system for testing the possibility of intracellular delivery of poly-nucleotides, in contrast to anti-sense oligo-nucleotides, which are generally required in a large excess relative to their target.

Liposome encapsulation is known to protect molecules against enzymatic degradation, and the encapsulation of poly IC in liposomes taken up non-specifically in vivo and in vitro has been reported to augment the efficiency of action of these molecules [12–14]. More

Abbreviations: IFN, interferon; dsRNA, double-stranded RNA; VSV, vesicular stomatitis virus; poly IC, the RNA duplex poly(rI) · poly(rC); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FCS, fetal calf serum; mAb, monoclonal antibody; MHC, major histocompatibility complex.

Correspondence: L. Leserman, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9, France.

recently, techniques have been developed to attach various protein ligands to the liposome surface, which permits a marked augmentation of the fixation of the liposomes to cells expressing a determinant for which the ligand is specific [15]. Depending on the ligand and the cell expressing it, the fixation of liposomes may be followed by their uptake and release of encapsulated contents intracellularly. Certain liposome-encapsulated drugs survive intracellular delivery and may exert marked, cell-specific, pharmacologic effects [16,17]. In the present study, we asked whether poly IC encapsulated in antibody-targeted liposomes would demonstrate the same synergistic interaction with IFN as free poly IC. We find that this is the case; antibody-targeted liposomes are at least three logs more efficient than free poly IC, and act according to the specificity of the targeting antibody.

Materials and Methods

Cell line and virus

Murine L929 cells (American Type Culture Colection, Rockville, MD, USA, Ref. CCL 1) were used throughout this work. They were grown in RPMI 1640 medium (Gibco, Cergy Pontoise, France) supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics. The Indiana strain of vesicular stomatitis virus (VSV) was produced and titrated in L929 cells.

Interferon and double-stranded RNA

Murine interferon α/β (IFN α/β), a generous gift from Dr. G. Rossi (Istituto Superiore di Sanita, Rome), was titrated with respect to a reference standard IFN α/β (Sigma Chemical Co., Lyon, France). Murine IFN γ was kindly provided by Dr. A.-M. Schmitt-Verhulst (Centre d'Immunologie INSERM-CNRS de Marseille-Luminy). IFNs were diluted to $4 \cdot 10^5$ units/ml with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline (pH 7.4) containing 2 mg/ml bovine serum albumin, then aliquoted and frozen at $-70\,^{\circ}C$ until use.

A high molecular weight complex of poly IC (Pharmacia, Uppsala, Sweden) (2 mg/ml) was sonicated (Branson, Danbury ,CT, USA, 20 Watts, 10 times 30 s) until a mean length of 500–600 bse pairs was obtained, as verified by 2% (w/v) agarose electrophoresis (buffer, 89 mM Tris, 89 mM borate, 1 mM EDTA (pH 7.5), 0.5 μg/ml ethidium bromide). Poly IC was labeled using polynucleotide kinase, according to standard protocols, to a final specific activity of $5 \cdot 10^5$ Bq/μmol.

Preparation of liposomes

Liposomes composed of 80 μmol total lipid (65% dipalmitoylphosphatidylcholine, 34% cholesterol (both from Sigma), and 1% *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia)-modified phosphatidylethanolamine (Sigma)) of 1000 Å nominal diameter, containing 2 mg/ml sonicated poly IC and/or 10 mM carboxyfluorescein (Kodak, Rochester, NY, purified as described [18]) in 145 mM NaCl, 10 mM Hepes, 1 mM EGTA (pH 7.45) were prepared using an 'Extruder' (Lipex Biomembranes, Inc., Vancouver, Canada), mounted with 0.1 μm polycarbonate filters (Nucleopore, Pleasanton, CA, USA), according to the manufacturer's instructions and published references [19] and were covalently coupled to *Staphylococcus aureus* protein A (Pharmacia), as described previously [15]. Uncoupled protein A, and unencapsulated poly IC were separated from liposomes on a Sepharose 4B (Pharmacia) column after treatment with 50 μg RNAse A (Sigma). We re-electrophoresed detergent-lysed liposomes, and confirmed by autoradiography that the encapsulated poly IC corresponded to the same length distribution as the material obtained after sonication. These studies suggested that the extrusion technique used did not influence the structure of liposome-encapsulated poly IC.

Antibodies

Murine IgG2a, κ monoclonal antibodies (mAbs) were purified from supernatant fluids of cultured hybridoma cells on protein A Sepharose (Pharmacia) columns. The target specificity of these mAbs is the murine major histocompatibility complex (MHC)-encoded H-2K molecule, for H100.5.28, and the human MHC-encoded HLA-B and C molecules for B1.23.2; the latter antibody is immunologically non-cross-reactive with the former antigen. The use of these protein A-binding mAbs in conjunction with protein A-bearing liposomes has been reported previously [20].

Liposome binding to L929 cells

10^6 L929 cells were incubated in medium alone, with 100 U/ml of γ IFN, or with 800 U/ml of α/β IFN for 16 h at 37 °C, then were washed and incubated with 5 μg/ml of anti-H-2K or isotype-matched control (anti-HLA) mAbs and carboxyfluorescein-containing protein A liposomes. After 1 h of incubation at 4 °C, cells were washed, and fixed with 2% formaldehyde. Cell fluorescence was analyzed by flow microfluorimetry, using an ODAM (Wissembourgh, France) flow cytometer.

Induction of IFN

IFN production was evaluated by modification of published protocols [21]. L929 cells were seeded at 10^5 cells/ml per well in 24 well tissue culture dishes and exposed to 800 U/ml IFN α/β, for 8 h. This step is defined as 'priming' [22]. Various concentraitons of free or liposome-encapsulated poly IC were then added, together with 5 μg/ml of the anti-H-2K or anti-HLA mAbs, and the dishes incubated for a further 2h at 37 °C. Cell monolayers were washed twice with RPMI 1640 supplemented with 10% (v/v) FCS, and incubated

at 37°C for a further 18–20 h. 500 μl of supernatant fluids were transferred to wells containing 10^5 L929 cells/ml in 24 well dishes. 18–20 h later the supernatant fluids were withdrawn and the culture challenged with 10^5 IU/ml VSV in freshly prepared medium containing 3% FCS. Incubation continued for 24 h at 37°C and the virus was titrated.

Virus titration

Dishes containing L929 and virus were frozen and thawed twice, and the VSV titres measured by endpoint dilutions of L929 cells seeded in 96 well tissue culture dishes. The number of infectious units (IU/ml) is estimated by the maximum likelihood method [23]. Because of the numerous cell treatments leading to induction of IFN, only differences of one log or more in virus inhibition should be considered as significant.

Toxic effect of free or liposome-encapsulated poly IC

In parallel experiments, L929 cells were primed with α/β IFN as indicated above, with 100 U/ml IFN γ for 8–16 h, or were left unprimed. After incubation with free or liposome-encapsulated poly IC and mAbs for various periods the effect on the cells' viability was evaluated by an MTT assay [24]. In this assay cells were incubated with 500 μg/ml of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h at 37°C, then the formazan product, which is dependent on enzymatic reduction by living cells, is solubilized in isopropanol and measured spectrophotometrically by an automated plate reader at 570 nm.

Results

Efficiency of the targeting of encapsulated poly IC

Unprimed L929 cells or cells pre-incubated with IFN α/β or γ were labeled with protein A-bearing liposomes containing carboxyfluorescein, in the presence of relevant (anti-H-2K) or control (anti-HLA), protein A-binding mAbs. As shown in Table I, cells incubated with the control mAb failed to bind the liposomes, but when incubated with the relevant mAb, liposomes were specifically bound. The binding was increased by both IFNs, as already reported [25], as a consequence of the increased expression at the surface of the target MHC molecules [26].

We next evaluated the effect of targeted liposomes containing poly IC on unprimed and IFN α/β primed cells. As shown in Fig. 1, supernatant fluids of cells primed with IFN α/β, and incubated with various concentrations of free or anti-H-2K targeted liposome-encapsulated poly IC were able to protect primed L929 cells from VSV infection. This protection was not observed when primed cells were incubated with liposomes containing poly IC and the control anti-HLA mAb. The anti-H-2K mAb, alone or in conjunction with liposomes containing carboxyfluorescein, had no effect, nor was the dose response curve of free poly IC (sonicated or not) changed by the presence of mAb-targeted empty liposomes (results not shown).

TABLE I

Binding of antibody-targeted liposomes to L929 cells

10^6 L929 cells were incubated in medium alone, with 100 U/ml of γ IFN, or with 800 U/ml of α/β IFN for 16 h, then were washed and incubated with 5 μg/ml of relevant (anti-H-2K) or isotype-matched irrelevant (anti-HLA) antibodies and carboxyfluorescein-containing protein A liposomes. After 1 h of incubation at 4°C, cells were washed, fixed with 2% formaldehyde, and analyzed by flow cytometry. The number refers to the mean fluorescence for 10000 cells, relative to the level (fixed at 1; arbitrary units) of intrinsic fluorescence (autofluorescence) of cells alone.

Antibodies	Without interferon	With γ interferon	With α/β interferon
Anti-H-2K	82	250	220
Anti-HLA	1	2	2

We confirmed that the antiviral activity induced by liposome-encapsulated poly IC was due to IFN α/β secretion, since it was completely neutralized when supernatant fluids were incubated with a rabbit anti-murine α/β IFN antibody 2 h before the transfer of the fluid supernatant to unprimed cells (data not shown).

Poly IC encapsulated in liposomes is resistant to degradative enzymes

There is a strong correlation between RNAse resistance of IFN-inducing polynucleotides and their activity [27]. Encapsulation in liposomes has been reported

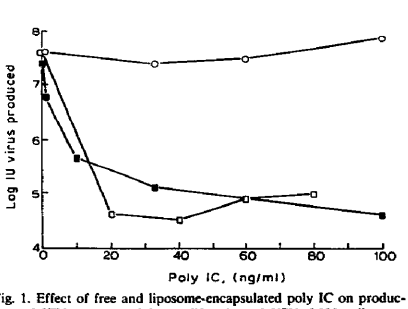

Fig. 1. Effect of free and liposome-encapsulated poly IC on production of IFN as assessed by proliferation of VSV. L929 cells were treated with α/β IFN for 8 h and then with free (■) or liposome-encapsulated poly IC for 2 h, in the presence of 5 μg/ml of relevant (anti-H-2K) (□) or isotype-matched irrelevant (anti-HLA) (○) antibodies. After washing, cells were incubated 18 h. Supernatant fluids were transferred to fresh L929 cells, which were then challenged with VSV. The numbers refer to the mean log virus titre for duplicate cultures of a representative experiment.

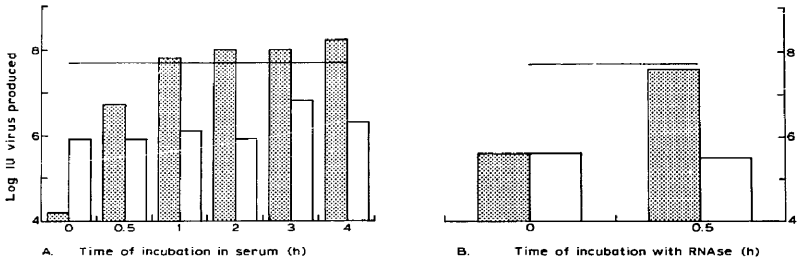

Fig. 2. Effect of cell supernatant fluids or RNAse A on free or encapsulated poly IC. (A) 50 ng/ml of free or encapsulated poly IC was incubated for various periods of time in supernatant fluids of confluent L929 cells cultured in medium containing 10% FCS and the capacity of these supernatants to induce IFN on primed cells was examined by inhibition of VSV replication. (B) 50 ng/ml of free or encapsulated poly IC was incubated with medium containing 100 μg/ml RNAse A (Sigma, St. Louis, MO) and 30 min later the mixture was evaluated for antiviral-inducing activity. ⊟, free poly IC; □, liposomal poly IC in the presence of anti-H-2K mAb; ———, level of control virus production for cells without poly IC.

to protect poly IC from degradation [14]. In order to demonstrate the protection of poly IC encapsulated in liposomes in this system, two experiments were performed. In the first, free or encapsulated poly IC was incubated for various periods of time in supernatant fluids of confluent L929 cells cultured in medium containing FCS and the capacity of these mixtures to induce IFN was examined. As indicated in Fig. 2A, free poly IC lost its inducing capability in less than 1 h, while encapsulated poly IC retained activity for at least 4 h. In a second experiment free or encapsulated poly IC was incubated with RNAse A and 30 min later the mixture was evaluated for antiviral-inducing activity. Liposomes retained their inducing capability; free poly IC did not (Fig. 2B).

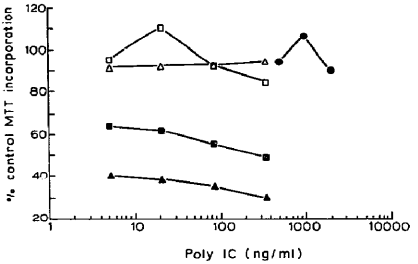

Fig. 3. Effect of free and liposome-encapsulated poly IC on the viability of L929 cells. 10^4 L929 cells in 100 μl were incubated with various concentrations of poly IC in liposomes targeted by the relevant (anti-H-2K) antibody after pre-incubation in medium only (□), with 100 U/ml γ IFN (■), or with 700 U/ml α/β IFN (▲). Other cells preincubated in α/β IFN were incubated with free poly IC (●) or with poly IC in liposomes targeted by an irrelevant (anti-HLA) antibody (△). After 24–48 h incubation MTT was added to duplicate wells, and its incorporation measured for duplicate samples after an additional 2-h incubation.

Toxic effect of liposome-encapsulated poly IC

L929 cells were incubated with various concentrations of poly IC in liposomes targeted by the relevant (anti-H-2K) mAb after preincubation in medium only, with γ IFN, or with α/β IFN. Other L929 cells pre-incubated with α/β IFN were incubated with free poly IC or with poly IC in liposomes targeted by the control (anti-HLA) antibody. As shown in Fig. 3, cells were killed by poly IC-containing liposomes only when targeted by anti-H-2K mAb and only when primed by

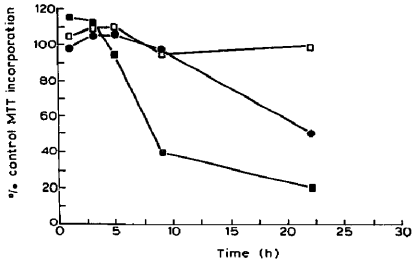

Fig. 4. Kinetics of cell death after incubation with liposomal poly IC. 10^4 L929 cells in 100 μl were incubated with 150 ng/ml of poly IC in liposomes targeted by the relevant (anti-H-2K) antibody after pre-incubation in medium only (□), with 100 U/ml γ IFN (●), or with 700 U/ml α/β IFN (■). At the intervals indicated, MTT was added to duplicate wells, and its incorporation measured after 2 h.

IFN. No toxicity was observed with free poly IC (sonicated or not), even at concentrations as high as 2 μg/ml. No toxicity was seen when primed cells were incubated with the anti-H-2K mAb and liposomes containing carboxyfluorescein only. In other experiments no toxicity of free poly IC was observed in the absence of IFN priming, and the mixture of free poly IC and empty, anti-H-2K antibody-targeted liposomes with α/β IFN-primed cells did not augment the toxicity of free poly IC (not shown).

We further evaluated the kinetics of cell death. As shown in Fig. 4, α/β IFN primed cells showed a rapid reduction of MTT incorporation (which correlated with cell death, indicated by Trypan blue uptake); 50% of the signal was lost in about 8 h. Cell death for γ IFN primed cells was of slower onset.

Discussion

Encapsulation of poly IC RNA in liposomes bound via monoclonal antibodies to cell-surface determinants and its incubation with cells in vitro resulted in IFN production, as measured by an inhibition of virus replication and a toxic effect on the targeted cells. Though liposomes bound to the H-2K molecule, known to be endocytosed by L929 and other L cell sublines [28,29], efficiently deliver encapsulated methotrexate into IFN primed and unprimed L929 cells (our unpublished results), action of liposome-encapsulated poly IC depended on priming, most effectively by α/β, or to a lesser extent, γ IFN. This is in agreement with the requirement seen when IFN production is induced by free poly IC [27], and suggests that the action of liposome-encapsulated poly IC depends, as does that of free poly IC, on IFN-inducible factor(s), such as (2′-5′)A synthetase, dsRNA-dependent kinase, and perhaps one or more of the other proteins known to be induced by the priming effect of the IFNs [6]. Increased expression of the target molecule of the monoclonal antibodies, which is the H-2K molecule, is also induced by the IFNs (Table I, Ref. 25); however, IFN γ was as effective for the induction of H-2K as IFN α/β, but was less effective for the pharmacologic effect, which indicates that increased level of liposome binding does not solely account for the pharmacologic effect of liposome-encapsulated poly IC.

IFN production was roughly equivalent after priming and incubation with either free or targeted liposome-encapsulated poly IC, though the toxic effect seen with poly IC in liposomes could not be detected, even at concentrations of free poly IC three logs higher. The lack of a marked enhancing effect of liposome-encapsulated poly IC on IFN production is consistent with the toxic effect of this reagent. The cells would probably have produced large amount of IFN had they survived, but the rapid toxicity seen (50% of the cells were killed in about 8 h, Fig. 4) prevented this synthesis. The toxicity seen clearly depended on IFN priming, and could be related to the introduction of a much larger amount of poly IC in liposomes than can be supplied in the free form. Qualitative differences would also be expected if encapsulated RNA, by virtue of its resistance to degradation (Fig. 2), entered into cells over a longer period, or was released into a different cellular compartment than the free molecule. Both liposome-encapsulated and free poly IC are taken up by an endocytic process, as confirmed by an inhibitory effect on their action by NH_4Cl (data not shown). (The question of whether they enter in the same endocytic vesicles or of at which point they leave endocytic vesicles cannot be directly addressed, in so far as the biological action of intracellular poly IC can be observed at levels below the sensitivity of biochemical or immunologic assays).

Our previous studies indicated both production of IFN and a toxic effect in HeLa cells microinjected with poly IC [9]; however, in that study the cell damage associated with the microinjection technique, and a variability of the site of injection from cell to cell rendered interpretation difficult, which motivated in part the present study. Here, all cells express the target molecule and bind comparable numbers of lipsoomes, which enter by the same endocytic pathway. We are aware that numerous problems of interpretation are also possible in the use of liposomes, including, but not limited to, effects of the antibodies or lipid used [30], and potential endotoxin contamination of liposomes, which may be difficult to detect [31]. As an example, some of the in vivo effects observed after administration of liposome-encapsulated poly IC were also seen after the injection of empty liposomes [32]. We therefore evaluated the same liposomes, varying the antibody, or the same antibody, varying the liposomes, in all experiments, and consequently feel that the demonstration of the intracellular entry of biologically active poly IC from antibody-targeted liposomes is unambiguous. An extension of studies using poly IC in targeted liposomes to a larger panel of normal and transformed cells will be necessary to determine if these effects are generalizable to other cells. An additional series of experiments is underway to evaluate the possibility of intracellular delivery of anti-sense DNA, using similar methods.

The toxicity of high concentrations of poly IC on certain tumor cell lines, including the L929 cells used in this study, has been noted previously [5], leading to evaluation of poly IC for the therapy of cancer, but the side effects of toxicity resulted in the cessation of those trials. A dsRNA of different structure, the mismatch form poly(rI). poly($rC_{12}U$) (Ampligen®) is currently under clinical evaluation in certain forms of cancer and in the prevention of clinical disease in individuals seropositive for the HIV 1 virus [33]. This form of RNA is well tolerated, but requires parenteral administration twice

or three times weekly. It will be of interest to determine whether effects pharmacologically equivalent to those induced by poly IC can be achieved in vitro with Ampligen in liposomes, and if so, whether the reagent is active in vivo at lower dose levels, or at less frequent intervals of administration than the free form.

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