

Mechanistic Studies of Polyene Enhancement of Interferon Production by Polyriboinosinic-Polyribocytidylic Acid

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The production of interferon by polyriboinosinic-polyribocytidylic acid [poly(I) · poly(C)] and poly(I) · poly(C)-diethylaminoethyl-dextran in L929 cells was enhanced from 10 to 100 times by polyene macrolides, including amphotericin B (AmB), AmB methyl ester, nystatin, and filipin. AmB and its water-soluble methyl ester were the most effective; retinol, a nonmacrolide polyene, was ineffective. Interferon induction by Newcastle disease virus was not enhanced by AmB. The kinetics of interferon production were not markedly altered by AmB. Polyenes and poly(I) · poly(C)-diethylaminoethyl-dextran did not need to be present on cells simultaneously to enhance interferon production. Pretreatment with polyenes was as effective as simultaneous addition. Even treatment of washed cells, several hours after removal of poly(I) · poly(C)-diethylaminoethyl-dextran, resulted in enhancement of interferon production. AmB did not appear to form a macromolecular complex with poly(I) · poly(C) in that neither the ultraviolet absorption spectrum nor the melting point of poly(I) · poly(C) was altered by mixing with AmB. Isotopic studies indicated that AmB did not enhance binding of poly(I) · poly(C) to cells. Since the macrolide polyenes have been demonstrated to bind to cell membrane sterols with subsequent alterations in membrane permeability barriers, they may enhance interferon production by increasing cell penetration of poly(I) · poly(C).

Amphotericin B (AmB), one of several polyene antibiotics, binds both fungal and vertebrate cell sterols and causes alterations in membrane permeability (1, 11, 12). The altered membrane permeability, which may be the primary mechanism of antifungal action, selectively favored penetration of anionic compounds (1, 9). After treatment with polyenes, enhanced uptake and action of nonpolyene antibiotics have been observed in a variety of eukaryotic cells, including L929 and 3T3 mouse fibroblasts (14, 15, 17). Inhibition of macromolecular synthesis by tetracycline, actinomycin D, and rifampin was potentiated by AmB, as was inhibition of HeLa and AKR mouse leukemia cell multiplication by actinomycin D and 1,3-bis(2-chloroethyl)-1-nitrosourea (14-18; G. Medoff, M. N. Goldstein, D. Schlessinger, and G. S. Kobayashi, *Clin. Res.* 23:425, 1975).

The enhancement of interferon production after polyriboinosinic-polyribocytidylic acid [poly(I) · poly(C)] and poly(I) · poly(C)-diethylaminoethyl (DEAE)-dextran resulted from AmB treatment of L929 cells (4). Interferon

production by L929 cells after poly(I) · poly(C) has generally been reported as minimal. However, after treatment of cells with 10 µg of AmB per ml, interferon production occurred at a relatively low poly(I) · poly(C) concentration (25 µg/ml), despite an increase in cytotoxicity, which occurred with the combination of poly(I) · poly(C) and AmB (4). The experiments described below were undertaken to delineate the mechanism by which AmB enhanced interferon production in L929 cells after poly(I) · poly(C).

MATERIALS AND METHODS

Cells. L929 mouse fibroblasts (CCL1, American Type Culture Collection) were grown as monolayers in Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 µg of gentamicin per ml (growth medium) in an incubator humidified with 5% CO₂. L929 cells were used for both interferon induction and assay.

Virus. Vesicular stomatitis virus, Indiana serotype, was propagated in L929 cells by infection at a multiplicity of 0.1, absorption for 1 h at 37°C, and harvest of the supernatant at 24 h. Newcastle disease virus (NDV), Hickman strain, was propagated in embryonated eggs. It was a gift from June Osborn and was

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kindly titrated by Robert Hanson in chicken embryo fibroblasts.

Polynucleotides. Poly(I)·poly(C) (Miles Laboratories, Inc., Elkhart, Ind.), which had a sedimentation coefficient of 11S, was dissolved in 0.2 M phosphate-buffered saline, pH 7.2. DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.) was combined with poly(I)·poly(C) at room temperature. The ultraviolet absorption spectrum of poly(I)·poly(C) was determined at room temperature in a Beckman model 25 scanning spectrophotometer. The melting point of poly(I)·poly(C) was determined, as previously described (22), in an automatic spectrophotometer equipped with dual thermospacers. The temperature in the cuvette compartment was calibrated with a linear thermosensor and was raised approximately 1°C/3 to 4 min. Readings of optical density were made manually to maximize sensitivity and reproducibility. [³H]poly(C)·poly(I) (specific activity, 17 μCi/mmol of phosphate) and [³H]poly(I)·poly(C) (specific activity, 26 μCi/mmol of phosphate) (Schwartz Bio Research, Inc., Orangeburg, N.Y.) were used for isotopic studies. Labeled duplexes were prepared by allowing appropriate single-stranded polyribonucleotides to anneal during cooling from 70 to 37°C. Annealed polyribonucleotides were dialyzed against a large volume of phosphate-buffered saline before use.

Polyenes. AmB (Fungizone, Grand Island Biological Co.) was diluted in Earle balanced salt solution (EBSS). Fungizone contains 41 mg of sodium deoxycholate for each 50 mg of AmB, but weights used in this paper refer to AmB, not Fungizone. Solvents for other polyenes were 0.5% Me₂SO for pure AmB type I (a gift of E. R. Squibb & Sons, Princeton, N.J.), EBSS for the methyl ester of AmB (AmBME; a gift of E. R. Squibb & Sons), 10% polyethylene glycol for nystatin (Calbiochem, La Jolla, Calif.), 0.5% ethanol for filipin (a gift of The Upjohn Co., Kalamazoo, Mich.), and retinol (Sigma Chemical Co.). In all cases polyenes were tested at a range of concentrations up to those resulting in gross microscopic cytotoxicity.

Interferon induction. Confluent cell monolayers were washed once with EBSS, overlaid with either poly(I)·poly(C) or poly(I)·poly(C)-DEAE-dextran, and incubated for 1 h at 37°C. The inducer was then aspirated, cells were washed three times with EBSS, and minimum essential medium containing 2% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml was added (maintenance medium). Media were collected for interferon assay after 24 h at 37°C. In those experiments in which polyenes were present either before or after induction, cells were washed three times with EBSS after the polyene-containing medium was removed. In experiments with NDV, cells were pretreated with AmB, washed once with EBSS, overlaid with NDV (multiplicity of infection = 1), and incubated for 1 h at 37°C. NDV was then aspirated, cells were washed four times with EBSS, and maintenance medium was added. After harvesting, interferon samples were treated with 0.1 volume of 1.5 M perchloric acid for 16 h and then centrifuged for 15 min at 11,000 × *g*. The supernatant was dialyzed exhaustively against EBSS and then diluted with an equal volume of maintenance medium for interferon assay. For isotopic studies, cells were grown in 16-mm

wells, exposed to tritiated poly(I)·poly(C) for 1 h, washed four times thereafter with EBSS, and trypsinized from the surface into siliconized centrifuge tubes. Cell-bound radioactivity was separated from that which had nonspecifically stuck to the dish surface by centrifugation at 400 × *g* for 10 min. The cell pellet was washed twice more with EBSS and solubilized in Unisol plus Unisol complement (Isolab, Akron, Ohio), Scintisol Complete was added, and activity was determined in a liquid scintillation counter. Identical results were obtained when cells were scraped from the surface rather than trypsinized.

All results presented are representative of those obtained in repeated experiments.

Interferon assay. Interferon was assayed by a semi-micro modification of the dye uptake method (5, 10). Twofold dilutions of each interferon sample were added in triplicate to confluent L929 cells. After 24 h at 37°C, interferon was aspirated, and the cells were washed with EBSS and infected with vesicular stomatitis virus (multiplicity of infection = 0.1). After 48 h, the medium was aspirated, and cells were incubated with neutral red (33 μg/ml) in EBSS for 75 min at 37°C. Monolayers were then washed with EBSS, and the dye was eluted into a 0.05 M NaH₂PO₄-50% ethanol mixture. Eluted dye was measured at 540 nm. The reciprocal of that interferon dilution at which uptake of dye was 50% of controls was considered the sample titer. Interferon titer was standardized and expressed as international reference units by incorporation of a mouse L929 cell laboratory standard into each assay and repeated comparison with the international mouse interferon reference standard.

RESULTS

Enhancement of interferon production by polyenes. Maximal enhancement of interferon production, induced by poly(I)·poly(C), occurred at an AmB concentration of 20 to 25 μg/ml (4). This concentration of AmB alone did not result in interferon production. The amount of interferon produced and the percentage of enhancement varied with the specific experiment, but a 100-fold increase in interferon titer at optimal AmB concentration was not uncommon. Interferon production by both poly(I)·poly(C) and poly(I)·poly(C)-DEAE-dextran was augmented by AmB (4). Enhancement occurred at concentrations of AmB as low as 0.5 μg/ml (Table 1). The enhancement of interferon production was not limited to the deoxycholate preparation of AmB. Pure AmB, which is poorly water soluble, was effective when solubilized in 0.5% Me₂SO. Water-soluble AmBME was also effective in increasing interferon production. Nystatin and filipin, other macrolide polyenes, also increased interferon production, although they were consistently less effective than AmB and AmBME (Table 1). Retinol (vitamin A), a polyene of unsaturated, straight-chain structure rather than the macrolide ring

TABLE 1. Enhancement of interferon production by various polyenes in L929 cells

Polyene concn ($\mu\text{g/ml}$)	Interferon produced with individual polyenes ^a					
	AmB (deoxycholate)	AmB (pure)	AmBME	Nystatin	Filipin	Retinol
0	10		70	20	90	<10
0.1					120	
0.5	60				170	
1.0	70	500			130	
5.0	190	860	110		26	
10	350	40	150			
20	800	<10	290			<10
50		<10	600	70		<10
100			130	120		<10
200				260		<10
500				330		<10
1,000				<10		
AmB 20 ^b	800	820	550	770	370	400

^a Each polyene was incubated with L929 cells at the indicated concentration for 24 h (except pure AmB, which was incubated for 1 h) before addition of 50 μg of poly(I) · poly(C) per ml–100 μg of DEAE-dextran per ml. Interferon amounts expressed in mouse interferon international reference units.

^b Concentration of AmB (deoxycholate), which was tested concurrently with other polyenes in each individual experiment.

structure of the polyene antibiotics, did not enhance interferon production at concentrations of 10 to 100 $\mu\text{g/ml}$, which were effective in inducing morphological changes in cells. None of the solvents used for the polyenes significantly altered interferon production by poly(I) · poly(C).

In contrast to interferon induction by poly(I) · poly(C), AmB did not enhance interferon production stimulated by infection with NDV. L929 cells, pretreated with AmB (20 $\mu\text{g/ml}$) for 24 h and then induced with NDV, produced an equivalent amount of interferon (2,900 U) as that produced by control NDV-infected monolayers (2,800 U).

Kinetic factors in enhancement of interferon production by polyenes. Media from cells, exposed to poly(I) · poly(C)–DEAE-dextran with and without AmB for various times, were collected for interferon assay. AmB-treated cells produced more interferon during all time periods (Fig. 1). AmB neither shortened nor prolonged the duration of interferon production. However, during the initial intervals of harvest, AmB-treated cells produced a somewhat lesser percentage of their total cumulative interferon when compared with control cells. By 16 h the cumulative percentage of interferon produced was equivalent. This slight delay in interferon production in AmB-treated cells may reflect the increased cellular toxicity associated with the combined use of AmB and poly(I) · poly(C) (4).

AmB and poly(I) · poly(C) did not need to be present on cells simultaneously to enhance interferon production. Pretreatment with polyenes, followed by cell washing before the addi-

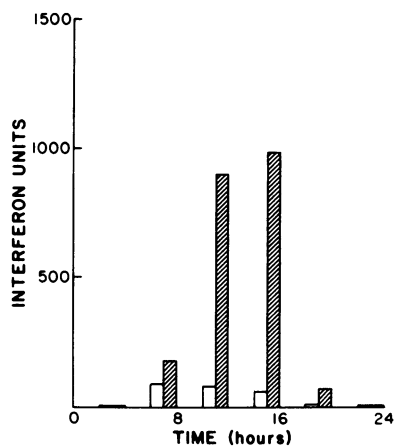


FIG. 1. Kinetics of interferon production after a single 1-h exposure to AmB and 50 μg of poly(I) · poly(C) per ml–100 μg of DEAE-dextran per ml. Interferon produced during various intervals without (open bars) and with (hatched bars) AmB (20 $\mu\text{g/ml}$). AmB and poly(I) · poly(C)–DEAE-dextran were added to cells at zero time. After 1 h, cells were washed three times in EBSS and minimum essential medium with 2% fetal calf serum added. The medium was harvested for interferon assay at the indicated times, cells were again washed twice, and media were readed.

tion of poly(I) · poly(C)–DEAE-dextran, was effective in enhancing interferon production as simultaneous addition (Table 2). Even treatment of cells with polyenes, several hours after removal of poly(I) · poly(C)–DEAE-dextran from the media, resulted in an enhancement of interferon production (Table 3). L929 cells were

treated with AmBME either simultaneously with or 8 h after poly(I) · poly(C)-DEAE-dextran. In monolayers treated with AmBME and poly(I) · poly(C)-DEAE-dextran simultaneously, interferon production peaked between 8 and 22 h and was essentially complete at the end of that interval. Cells treated with AmBME 7 h after the removal of poly(I) · poly(C)-DEAE-dextran and the washing of the cells three times also produced similarly increased total levels of interferon. However, late addition of AmBME resulted in a delay in peak interferon production to between 14 and 32 h. Thus, the enhancing effect of polyenes was independent of the time of their addition, although the kinetics of interferon production could be markedly influenced.

Lack of macromolecular complex formation. That poly(I) · poly(C) and AmB did not need to be used concurrently suggested that no macromolecular complex formed between polyenes and poly(I) · poly(C). To verify this further, the ultraviolet absorption spectrum and the melting point of poly(I) · poly(C) were determined after mixing AmB and poly(I) · poly(C) at 37°C for 15 min. Although poly(I) · poly(C) and AmB had coincident absorption peaks, no shift or dampening of these peaks was apparent in the mixture (Fig. 2). Rather, an additive effect was observed. For comparison, a mixture of poly(I) · poly(C)-DEAE-dextran was treated similarly. A marked decrease in absorbance of the poly(I) · poly(C)-DEAE-dextran mixture re-

sulted, attributable to the formation and precipitation of the poly(I) · poly(C)-DEAE-dextran complex. AmB also had no effect on the melting temperature or melting curve of poly(I) · poly(C) (data not shown). Thus, AmB and poly(I) · poly(C) apparently did not interact directly in solution or on the cell surface.

Radiolabeled polyribonucleotide studies. Cells pretreated with AmB were exposed to tritiated poly(I) · poly(C), and cell-bound radioactivity was determined. In the short incubation periods used, little difference was observed between trichloroacetic acid-insoluble cell extracts and total cell radioactivity. Radioactivity was not increased in the AmB-treated cells com-

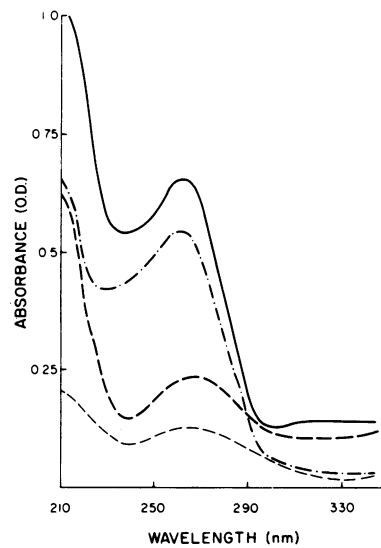


FIG. 2. Ultraviolet absorption spectrum of poly(I) · poly(C) and poly(I) · poly(C)-AmB mixture. Poly(I) · poly(C), 50 µg/ml (— · —); poly(I) · poly(C), 50 µg/ml with AmB, 20 µg/ml (---); and AmB, 20 µg/ml (—) were incubated for 15 min at 37°C in EBSS and centrifuged at 20,000 × g for 15 min, and the ultraviolet absorption spectra were determined. For contrast with the effects of AmB, 50 µg of poly(I) · poly(C) per ml was combined at room temperature for 30 min with 100 µg of DEAE-dextran per ml and then treated similarly (—).

TABLE 2. Effect of time of addition of poly(I) · poly(C) and AmB on interferon production

Duration (h) of AmB ^a	Interferon units
-24 to +1	3,200
-1 to 0	3,600
0 to +1	3,900
No AmB	240

^a A complex of poly(I) · poly(C)-DEAE-dextran was added to L929 cells at 0 time to final concentrations of 100 and 200 µg/ml, respectively. AmB was added for the times indicated at a concentration of 20 µg/ml. Cells were washed twice with EBSS after indicated treatments.

TABLE 3. Interferon induction after late addition of AmBME

Additions at: ^a	Interferon produced during following intervals (U/ml) ^b :					
	0 to 1 h	8 to 9 h	1 to 8 h	8 to 14 h	14 to 22 h	22 to 32 h
Poly(I) · poly(C)			<10	<10	<10	11
Poly(I) · poly(C) and AmBME			120	480	480	71
Poly(I) · poly(C)		EBSS	<10	<10	<10	13
Poly(I) · poly(C)		AmBME	<10	33	470	330

^a Concentrations of additions: poly(I) · poly(C), 50 µg/ml-DEAE-dextran, 100 µg/ml; AmBME, 50 µg/ml. Monolayers were washed three times with EBSS 1 h after all additions.

^b All intervals are expressed in relation to poly(I) · poly(C) addition at 0 h.

pared with control cells, regardless of whether poly(I) · poly(C) or poly(I) · poly(C)-DEAE-dextran was used as an inducer (Table 4). Furthermore, when the tritium label was on the poly(I) strand rather than the poly(C) strand, no differences in bound radioactivity between control and AmB-treated cells were observed.

DISCUSSION

Polyenes were effective even at relatively low concentrations in enhancing interferon production by both poly(I) · poly(C) and its macromolecular complex with DEAE-dextran. AmB, AmBME, nystatin, and filipin all increased interferon production, although retinol, a polyene without the macrolide structure, was ineffective in enhancing interferon production. The macrolide polyenes bind to cell membrane sterols and have resulted in membrane-permeability alterations (11, 12). A conceptual model of membrane interaction based on amphipathic properties of these compounds has been proposed (1). Enhancement by polyenes of the biological effect of another polyribonucleotide, encephalomyocarditis virus ribonucleic acid, occurred after cell treatment with AmBME (Booth and Borden, unpublished data).

The characteristics of enhancement of interferon production by polyenes were distinct in several respects from those of other compounds previously found to increase production of interferon by poly(I) · poly(C). Polycations, such as DEAE-dextran, neomycin, protamine, and poly-D-lysine, increased interferon production by poly(I) · poly(C) (3, 8, 13, 19). Polycations probably increased both cell binding and resistance to nucleases of poly(I) · poly(C) (2, 3, 6, 7, 13). Macromolecular-complex formation between the polyanion, poly(I) · poly(C), and polycations can be demonstrated (6, 13). Calcium also was recently found to enhance poly(I) · poly(C) binding to the cell, with a resulting increase in interferon production (Booth and Borden, *J. Gen.*

Virol., in press). Our data do not support a similar mechanism of action for the polyene antibiotics: (i) cells were treated with polyenes before, during, or after addition of poly(I) · poly(C) and produced increased amounts of interferon; (ii) polyenes augmented the enhancing effects of DEAE-dextran; (iii) AmB changed neither the melting temperature, melting curve, nor absorption spectrum of poly(I) · poly(C), suggesting the compounds did not directly interact; and (iv) AmB did not increase cellular binding of radiolabeled poly(I) · poly(C).

Interferon production has been enhanced and prolonged by appropriate use of inhibitors of protein synthesis (20, 21). This superinduction has been postulated to result from inhibition of synthesis of a control protein for interferon that is produced after the formation of interferon messenger ribonucleic acid. Polyenes probably do not act by a similar mechanism because, unlike the metabolic inhibitors, polyenes could be added before or during induction and did not change the late kinetics of interferon production, and at the concentrations used, AmB had little effect on protein and ribonucleic acid synthesis in L929 cells (15). The differences suggest that the macrolide antibiotics may increase interferon production by a unique mechanism.

Polyene enhancement of poly(I) · poly(C)-induced production appeared to involve an early step in the induction process. When AmBME was added several hours after removal of excess poly(I) · poly(C), interferon production was enhanced. The kinetics of the enhanced interferon response were similar to those observed after concomitant addition of poly(I) · poly(C) and polyene. The production of interferon was delayed just as though the actual induction had occurred at the time of the addition of the polyene rather than the poly(I) · poly(C). This suggested that the polyenes did not enhance interferon release but were potentiating the effects of previously cell-associated poly(I) · poly(C). Because AmB did not enhance interferon production induced by NDV, the polyenes must have affected an initial step in the interferon induction process not shared by the two inducers.

Polyenes probably either increased the penetration of poly(I) · poly(C) into the cell or increased the availability of a specific membrane binding site for interferon induction by poly(I) · poly(C). Membrane-permeability alterations, induced by polyenes, have enhanced cell penetration by anionic compounds and have potentiated intracellular action of metabolic inhibitors (1, 9, 14, 15, 17). On these bases, we favor a similar mechanism for the enhancement of interferon production by polyenes, with cell pen-

TABLE 4. Effect of AmB on cell binding of tritiated poly(I) · poly(C)^a

Inducer ^b	Poly(I) · poly(C) binding (% of input counts)	
	AmB (0 µg/ml)	AmB (20 µg/ml)
	[³ H]poly(C) · poly(I)	110(0.4)
[³ H]poly(C) · poly(I)- DEAE-dextran	13,500 (37)	9,700 (36)

^a The data on each inducer are representative of three experiments.

^b Concentrations: Poly(I) · poly(C), 50 µg/ml; DEAE-dextran, 100 µg/ml.

etration by polyribonucleotides being a critical step for interferon induction.

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