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Effects of Ionenes on Interferon Induction by Poly(Inosinic Acid)·Poly(Cytidylic Acid)

ERIK DE CLERCQ, BOREK JANIK,* AND RONALD G. SOMMER

*Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium; and Miles Laboratories, Inc., Elkart, Indiana 46514**

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Depending on the spacing of their positive charges, ionenes, a class of quaternary ammonium polymers, increased the interferon-inducing activity of poly(inosinic acid)·poly(cytidylic acid) in mouse L-929 cells, whereas they did not enhance poly(inosinic acid)·poly(cytidylic acid) induced interferon production in primary rabbit kidney and human skin fibrolast cells.

Many polyamines have been shown to enhance the formation of interferon and resistance to viral infections induced by poly(inosinic acid)·poly(cytidylic acid) [$(I)_n \cdot (C)_n$] in a variety of cell systems (4, 9, 27). Diethylaminoethyl (DEAE)-dextran, the most widely studied polyamine, was found effective in a variety of cells such as chicken embryo (3, 13, 15), mouse L-(1, 5-7, 17), primary rabbit kidney (PRK) (29), human leukocytes (8), and human skin fibroblasts (HSF) (22, 26). However, attempts to potentiate the $(I)_n \cdot (C)_n$-induced interferon response by DEAE-dextran and other polyamines did not prove successful in all cell systems (9, 16).

Ionenes, quaternary polyamines of the general formula $[N(CH_3)_2\text{-}(CH_2)_x\text{-}N(CH_3)_2\text{-}(CH_2)_y]_z^{2+}\ Br^-_{2z}$ (2), were found to exert biological activity in a number of systems (23). Polyamines structurally similar to ionenes can enhance antitumor activity of (11) and interferon induction by double-stranded mycophage ribonucleic acid (RNA). (The numbers in ionene names refer to the x and y multiples of the methylene group repeating unit. The values of intrinsic viscosity, $[\eta]$, in 0.4 M KBr for 3,3-, 6,6-, and 6,10-ionene were 0.115, 0.298, and 0.335, respectively. The correlation of $[\eta]$ with molecular weight (M) (2) gives a molecular weight $M = 4.2 \times 10^4$ for 6,6-ionene. Values of molecular weight for 3,3- and 6,10-ionene can only be estimated due to unavailability of correlation parameters (2) as being of the same order but lower than that for 6,6-ionene.) We now report enhancement of the $(I)_n \cdot (C)_n$-induced interferon response in L-cells treated with ionenes.

The ionenes tested for their effects on interferon induction included 3,3-ionene, 6,6-ionene, and 6,10-ionene (M. R. Harnden, A. G. Brown, T. J. Sharpe, and R. A. Vere-Hodge, Am. Chem. Soc., 166th Meet., Chicago, Ill., Abstr. no. MEDI 48, 1973).

Treatment of L-929 cells (Table 1) with 10 μg of 6,10-ionene per ml for 1 h before their exposure to $(I)_n \cdot (C)_n$ markedly increased ($P < 0.01$) the production of interferon. At the same concentration, neither 3,3-ionene, 6,6-ionene, nor DEAE-dextran showed any significance ($P <$ 0.05) stimulatory effect. At 100 μg/ml, however, DEAE-dextran showed any significant ($P <$ tion ($P < 0.01$) while 6,10-ionene was cytotoxic. None of the compounds tested were stimulatory at 1 μg/ml.

When a treatment regimen similar to that used in L-929 cells (Table 1) was applied to primary rabbit kidney (PRK) or human skin fibroblast (HSF) cells, neither ionenes nor DEAE-dextran caused an increased interferon response. In fact, such treatment with ionenes or DEAE-dextran for 1 h before the addition of $(I)_n \cdot (C)_n$ tended to decrease the subsequent production of interferon at both 10-μg/ml and 100-μg/ml concentrations of the individual components (data not shown).

Other treatment regimens were then installed in attempts to increase the interferon production in PRK and HSF cells. The ionenes or DEAE-dextran were directly mixed with $(I)_n \cdot (C)_n$ at a 1:1 (wt/wt) ratio, and after the cells had been incubated with these mixtures, cycloheximide and actinomycin D were added (10, 28) to further stimulate interferon production ("superinduction") (Table 2). In PRK cells, the only significant difference observed ($0.01 < P < 0.05$) was between the treatments with 6,6-ionene and 6,10-ionene, whereby the former slightly decreased and the latter slightly increased interferon production. In HSF cells, the 3,3- and 6,6-ionenes depressed ($0.01 < P < 0.05$) interferon production, whereas neither 6,10-io-

TABLE 1. *Effect of ionenes on interferon-inducing capacity of* $(I)_n \cdot (C)_n$ *in mouse L-929 cells*

Polycation	Interferon yield (U/ml)[a, b]						
	1[c]		10			100	
3,3-Ionene	6	3	3	3	9	19	20
6,6-Ionene	10	10	10	3	4	10	6
6,10-Ionene	16	150		186	1,700	T[d]	T[d]
DEAE-dextran	20	45	16	10	6	2,500	500
Control[e]	20	20	20	3	3	20	20

[a] Confluent L-929 cell monolayers were treated with the ionenes or DEAE-dextran (Pharmacia), diluted in serum-free minimal essential medium (MEM) to the indicated concentrations, for 1 h at 37°C, then washed (3×) with MEM and exposed to $(I)_n \cdot (C)_n$ (P-L Biochemicals) at 10 μg/ml in MEM for 1 h at 37°C. The cells were washed again (3×) with MEM and further incubated with MEM containing 3% calf serum for 24 h at 37°C. The supernatant fluids were then collected and titrated for interferon by a vesicular stomatitis virus plaque inhibition assay in L-929 cells. The data represent individual interferon titer readings from different replicate experiments.

[b] The $\log_{10}$ values of interferon yields were subjected to studentized range statistics to determine whether or not the individual treatments with polycations differ significantly from each other or from the control with respect to interferon yields. The statistical significance is noted in the text.

[c] Polycation concentration (micrograms per milliliter).

[d] T, At 100 μg/ml, 6,10-ionene was toxic for the cells.

[e] The control experiments were carried out as shown in *a* except in the absence of polycation.

TABLE 2. *Effect of ionenes on interferon-inducing capacity of* $(I)_n \cdot (C)_n$ *in primary rabbit kidney (PRK) and human skin fibroblast cells (HSF)*

Polycation	Interferon yield (U/ml)[a, b]								
	PRK						HSF		
3,3-Ionene	3,000	1,500	3,000	6,000	6,000	1,000	20	50	100
6,6-Ionene	1,000	1,000	1,000	2,000	1,000	2,000	20	60	200
6,10-Ionene	3,000	1,500	15,000	10,000	6,000	10,000	100	150	500
DEAE-dextran		1,000	6,000	3,000	1,000	10,000	200	600	4,000
Control[c]	3,000	1,500	3,000	1,000	1,000	10,000	300	600	5,000

[a] Ionenes or DEAE-dextran and $(I)_n \cdot (C)_n$ were mixed in serum-free minimal essential medium (MEM) to yield a final concentration of 10 μg/ml for each component. The mixtures were first incubated for 1 h at 37°C and then applied onto the cells for another 1 h at 37°C. The cells were washed (3×) with MEM and further processed according to a "superinduction" schedule, i.e., PRK cells were treated consecutively with cycloheximide (2 μg/ml) for 3 h and actinomycin D (3 μg/ml) for 0.5 h; HSF cells were treated with cycloheximide (10 μg/ml) for 6 h, and actinomycin D (1 μg/ml) was added for the last 2 h of this 6-h incubation period. After removal of the metabolic inhibitors, PRK and HSF cells were washed (3×) with MEM and further incubated with MEM containing 3% calf serum for 24 h at 37°C. The supernatant fluids were then collected and titrated for interferon by a vesicular stomatitis virus cytopathogenicity inhibition assay in PRK cells (rabbit interferon) or HSF cells (human interferon). The data represent individual interferon titer readings from replicate experiments.

[b] See footnote *b* in Table 1.

[c] See footnote *e* in Table 1.

nene nor DEAE-dextran affected it. Interferon induction was also monitored in HSF cells which had been "primed" with interferon (10) or exposed to ultraviolet irradiation (19) and in HSF cells which had been primed and "superinduced." In none of these systems, however, did mixtures of ionenes (or DEAE-dextran) with $(I)_n \cdot (C)_n$ (each component at 10 μg/ml) prove more efficacious in eliciting interferon production than $(I)_n \cdot (C)_n$ alone (data not shown).

The results presented herein establish that polycations such as 6,10-ionene and DEAE-dextran, while potentiating the induction of interferon by $(I)_n \cdot (C)_n$ in some cell cultures, fail to do so in others. They further indicate that the spacing or density of the positive charges in the ionene is of critical importance, since only 6,10-ionene and not 3,3- and 6,6-ionenes proved effective in enhancing the interferon induction by $(I)_n \cdot (C)_n$ in L-cells. Quarternary polyamines readily complex with double-stranded RNA (11). The efficacy of ionenes to enhance interferon induction seems to be related to their avidity to complex. We observed (data not shown) that 6,10-ionene precipitates various polynucleotides at concentrations severalfold lower than do 3,3- or 6,6-ionene.

Although the exact mechanism by which DEAE-dextran or other polyamines potentiate interferon production has not been elucidated, DEAE-dextran is assumed to act by promoting the interaction of $(I)_n \cdot (C)_n$ with its putative receptor site (13, 22). DEAE-dextran greatly enhances the rate of uptake of $(I)_n \cdot (C)_n$ by the cells (1, 3). Ionenes, too, exhibit uptake-stimulating properties, whereby 6,10-ionene is by far more effective than 6,6- and 3,3-ionenes (12). In neither case, however, has it been determined with certainty whether this uptake represents penetration of the polynucleotide across the cell membrane or only a cell surface adsorption. DEAE-dextran and quarternary polyamines also protect various polynucleotides against hydrolysis by endonucleases (7, 11, 12, 22), which can be present in the serum (14, 20, 25) and/or associated with the cells (24). In general, the stimulatory effects of polycations seem to be exerted through neutralization of the negative cell surface charges and consequent destabilization of the cell membrane (18, 21) and through interaction with polynucleotides which may result in reduction of their repulsion from the cell surface (17), stabilization of their conformation, and protection against enzymatic degradation (11, 17, 21). Why a polycation cannot enhance $(I)_n \cdot (C)_n$ induced interferon production in every cell line and why the induction can be enhanced by one polycation and not by its homologue remains to be answered. It appears that more delicate complementarity requirements have to be met than simply the presence of negative charges on the cell surface or polynucleotide inducer and positive charges on the polycation. Our findings warn against generalizations based on the results obtained in one particular cell system regarding the effects of polycations on the interferon-inducing activity of double-stranded RNAs and the mechanism(s) involved.

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