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Inhibition of RNA Synthesis in Human Lymphoid Cells Induces Interferon Production (41175)

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Abstract. Human lymphoid cell cultures produced an interferon-like activity in the absence of inducers. Using actinomycin D, cycloheximide, and other metabolic inhibitors, we have shown that the cellular requirement for *de novo* RNA synthesis is not a requisite for interferon induction. We show here that the production of an interferon-like protein may be triggered in cultured lymphoid cells by substances capable of suppressing DNA-dependent RNA synthesis. Our data suggest that a rapidly turning over regulatory mechanism controls the production of an interferon-like molecule in lymphoid cells. In fact, the induction by inhibitors of RNA synthesis and the requirement for protein synthesis for production, but not for induction, suggests a preformed messenger RNA for an interferon-like molecule whose translation is normally prevented by a rapidly turning over mechanism which requires ongoing RNA synthesis. Experiments with neutralizing antibody for interferon suggest a new antigenic type of immune interferon or a slightly different molecule with lowered affinity for antibody. The very existence, however, of such a rapidly activated regulation of interferon production infers an important role in lymphocyte response and immune regulation.

The three types of human interferon so far identified [fibroblast (HuIFN-β), leukocyte (HuIFN-α), and immune (HuIFN-γ)] have been characterized as *de novo* synthesized proteins whose production requires both RNA and protein synthesis by the induced cells (1). While studying specific mechanisms of interferon induction in lymphoid cell cultures (2), we have noted the appearance of interferon-like activity in cells treated with inhibitors of RNA synthesis. Therefore, the hypothesis that some interferon-like activity was being expressed in the absence of *de novo* RNA synthesis was considered.

Initial experiments showed that treatment of lymphoid cell cultures with actinomycin D, prior to addition of inducers of immune interferon, resulted in complete and permanent suppression of RNA synthesis. Interferon production was substantially reduced under these conditions but not completely inhibited. We have now extended this study through experiments which were designed to test whether in the absence of interferon inducers, actinomycin D and other inhibitors of macromolecular synthesis are also able to elicit interferon production from lymphoid cell cultures.

This study shows that the production of an interferon-like protein may be triggered, in cultures of human peripheral blood mononuclear cells, by substances capable of suppressing DNA-dependent RNA synthesis.

Materials and Methods. Lymphoid cell cultures (10^6 cells/ml) were established, as previously described, by Ficoll–Hypaque gradient sedimentation of peripheral blood from healthy adult donors (2). McCoy's 5A Medium supplemented with 10% (v/v) autologous serum or 1% human albumin was used for all lymphoid cell cultures.

Lymphoid cell cultures were treated with actinomycin D (Boeringer–Mannhein, 5 μg ml^{-1}), 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB, Calbiochem, 50 μg ml^{-1}), α-amanitine (Calbiochem, 5 μg ml^{-1}), mitomycin C (Sigma, 200 μg ml^{-1}), adenine arabinoside (Ara-A, PL Biochemicals, 1 μg ml^{-1}), cytosine arabinoside (Ara-C, PL Biochemicals, 1 μg ml^{-1}), bromodeoxyuridine (BUDR, Calbiochem, 1 μg ml^{-1}), cycloheximide (Sigma, 20 μg ml^{-1}), and fluorophenylalanine (FPA, Sigma, 800 μg

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ml^{-1}). The cultures were incubated at 37°, after 24 hr incubation the fluids were harvested and assayed for antiviral activity as previously described (2) using human WISH cell cultures and Sindbis virus as the challenge virus. Samples of human immune and leukocyte interferon containing 100 reference units were assayed at the same time as the samples under study.

Results. Table 1 shows that all the compounds capable of inhibiting DNA-dependent RNA synthesis (actinomycin D, α-amanitine, and DRB) caused the appearance of substantial levels of antiviral activity while in no instance did the cells treated with inhibitors of protein synthesis (cycloheximide and FPA) or with inhibitors of DNA synthesis (mitomycin C, Ara-A, Ara-C, and BUDR) produce detectable levels of antiviral activity. Control experiments carried out in parallel showed that the same dose of actinomycin D capable of eliciting the appearance of antiviral activity was able to irreversibly suppress RNA synthesis by 96% as measured by tritiated uridine incorporation into TCA precipitable material.

The data shown in Table I suggest that the appearance of the antiviral activity is triggered by the blockade of RNA synthesis. It remained to be established, however, whether the activity was due to the induction of newly formed antiviral substance(s) (AVS) or to the release of preformed molecule(s). To explore these two possibilities, lymphoid cell cultures were treated with actinomycin D to block RNA synthesis, or with cycloheximide and actinomycin D to block both protein and RNA synthesis. One hour later the inhibitors were removed by multiple washing and the cultures were refed with fresh medium or with medium containing cycloheximide or FPA. Under these conditions, RNA synthesis remained suppressed in every culture due to the irreversibility of antinomycin D action, whereas protein synthesis, where reversibly blocked by cycloheximide, could resume only in the cultures refed with inhibitor-free medium. Therefore, it was possible to determine whether ongoing protein synthesis was required for induction and production of AVS in cells treated with actinomycin D. The fluids were then harvested 24 hr later and assayed for AVS. The results of a representative experiment are shown in Table II.

It may be seen that the presence of cycloheximide during the 1-hr treatment with actinomycin D did not prevent the appearance of the AVS, while the addition of cycloheximide or FPA after treatment with actinomycin D did not inhibit the appearance of AVS. The data show, therefore, that protein synthesis is not required during the induction process, but it is needed thereafter, suggesting that the antiviral activity induced by actinomycin D requires the production of newly formed protein(s).

To study the kinetics of production of AVS, lymphoid cell cultures were treated with cycloheximide and actinomycin D, in-

TABLE I. APPEARANCE OF ANTIVIRAL ACTIVITY IN CULTURE FLUIDS OF HUMAN LYMPHOID CELLS TREATED WITH DIFFERENT INHIBITORS OF MACROMOLECULAR SYNTHESIS

Treatment	No. of experiments	Mean titer of antiviral activity (log)	SD
Actinomycin D	10	2.1	0.2
DRB	5	1.9	0.3
α-Amanitine	4	2.0	0.3
Cycloheximide	7	<1.0	0.0
FPA	5	<1.0	0.0
Mitomycin C	2	<1.0	0.0
Ara-A	2	<1.0	0.0
BUDR	2	<1.0	0.0
None	10	<1.0	0.0
Freezing–thawing	2	<1.0	0.0

Note. Cell cultures (10^6/ml) were treated as shown, incubated at 37°, after 24 hr the fluids were harvested and assayed for antiviral activity.

TABLE II. EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON ACTINOMYCIN D-INDUCED ANTIVIRAL ACTIVITY IN LYMPHOID CELL CULTURES

First treatment	Second treatment	Titer of AVS (log)
Actinomycin D	None	2.1
Actinomycin D plus cycloheximide	None	2.3
Actinomycin D	Cycloheximide	<1.0
Actintomycin D	FPA	<1.0
Actintomycin D plus cycloheximide	Cycloheximde	<1.0
Actinomycin D plus cycloheximide	FPA	<1.0

Note. Cell cultures were treated with inhibitor for 1 hr at 37°, washed, and refed with fresh medium or medium containing the second treatment. Culture fluids were harvested 24 hr later and assayed for antiviral activity.

cubated at 37° in a waterbath, 1 hr later the unbound inhibitors were removed by multiple washing in a refrigerated centrifuge. These cultures were then refed with fresh prewarmed medium and were reincubated at 37°. At preestablished times, samples were harvested for titration of the antiviral activity. The results of a representative experiment are shown in Fig. 1.

It can be seen that a substantial yield of antiviral activity was produced within 1 hr after the removal of cycloheximide and actinomycin D and that the peak titer was reached during the next 2 hr. Additional experiments revealed that the yield of AVS was unaffected by the addition of cordycepin (50 μg ml^{-1}), after removal of cycloheximide and actinomycin D.

Initial experiments showed that the AVS found in the samples had the characteristics of interferon: inactivation by proteolytic enzymes, lack of inactivation by ribonuclease, activity against different viruses, inactivity on heterologous cells, activity on different types of human cells, and block of action by actinomycin D and FPA. Further characterization experiments were carried out to compare the properties of the actinomycin D-induced AVS with the properties of the samples previously identified as human leukocyte and immune interferon. The results of these experiments are summarized in Table III.

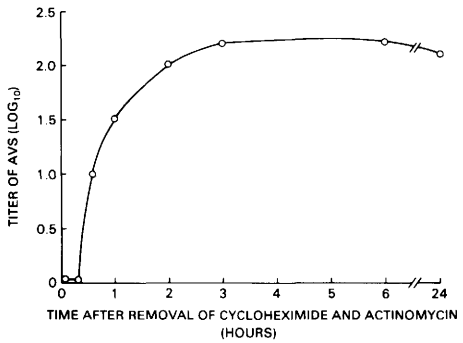

FIG. 1. Kinetics of production of actinomycin D-induced antiviral substance.

It may be seen that the AVS induced by actinomycin D shares with the immune interferon all the biological properties tested, instability at low pH, blockade by cycloheximide (3), and slow kinetics of activation of the cellular antiviral resistance (4), but it appears to be antigenically different from immune, fibroblast, and leukocyte interferon. The lack of neutralization by a combined treatment with antibody to leukocyte, fibroblast, and immune interferons rules out the possibility that the activity induced by actinomycin D would be due to a mixture of both types of interferon. The production of interferons with similar properties, i.e., with the characteristics of immune interferon but without antigenic recognition by antibody to the three currently recognized types of interferon has also been found in other laboratories (S. Baron and M. Langford, personal communication).

Discussion. Production of fibroepithelial interferon may be enhanced by treatment of certain types of cells with inhibitors of protein or RNA synthesis at critical times after induction by viruses or double-stranded RNA (5–9). Production of interferon (presumably fibroepithelial) may be triggered by inhibitors of protein and RNA

TABLE III. COMPARATIVE PROPERTIES OF ACTINOMYCIN D-INDUCED ACTIVITY, LEUKOCYTE INTERFERON, AND IMMUNE INTERFERON

Material tested	Percentage of antiviral activity expressed after:					
	1 hr treatment at pH 3.0	Treatment of the cells in the presence of cycloheximide	Treatment of the cells for 10 min	Pretreatment with antibody to leukocyte interferon[a]	Pretreatment with antibody to immune interferon[b]	Pretreatment with combined antibody to leukocyte and immune interferon
Antinomycin D-induced antiviral substance[c]	10	0	0	100	100	100
Leukocyte interferon[c]	100	100	50	0	100	0
Immune interferon[c]	0	0	0	100	0	0

[a] Kindly provided by Dr. K. Pauker. Neutralizing titer of 1/10,000 to leukocyte interferon and 1/1200 to fibroblast interferon.
[b] Neutralizing titer of 1/1000 to immune interferon/no neutralizing activity to leukocyte and fibroblast interferon. Rabbit antisera to human immune interferon kindly provided by Dr. John Stanton.
[c] 100 reference units were used in each experiment.

synthesis even in unstimulated cultures, although this event seems to occur only in certain nondiploid cell lines (10). Taken together these findings suggest that the production of fibroepithelial interferon is regulated by specific protein repressor(s). This type of regulation, however, has not yet been reported for leukocyte or immune interferon. The data reported in this study suggest that a rapidly turning over regulatory mechanism controls the production of an interferon-like molecule in lymphoid cells. In fact, the induction by inhibitors of RNA synthesis and the requirement of protein synthesis for production, but not for induction, suggests a preformed messenger RNA for an interferon-like molecule whose translation is normally prevented by a rapidly turning over mechanism which requires ongoing RNA synthesis. The presence of preformed mRNA in the cytoplasm is supported by the work of Berger *et al.* (11) and the finding that production of AVS is unaffected by cordycepin, a compound which specifically blocks transport of nuclear heterogeneous RNA to the cytoplasm. Perhaps this mechanism is analogous to the hemin translational control described in rabbit reticulocytes (12). Within this system, hemin binds to a protein repressor stopping the formation of a translational inhibitor, which prevents hemin production. If this analogy is correct, a translational inhibitor would normally prevent expression of the interferon message. Blockade of RNA synthesis may, therefore, terminate production of the message for the inhibitor. This implies that the inhibitor is very rapidly turning over. More studies are required to test these hypotheses. Alternative hypothesis may consider that interferon is first synthesized as a "pro" interferon and that blockade of cellular RNA would prevent the formation of a repressor protein allowing the "pro" interferon message to be translated.

The possibility that the interferon activity was due to endotoxin contamination of our samples was also considered and rejected for the following reasons. In order for endotoxin to cause this interferon production, both mRNA and protein synthesis would be necessary. It would have to be equally

superinduced by the three different translational inhibitors used in this study, and it would also have to be present in all of our healthy donors. In addition, comparison of the characteristics of our actinomycin D-induced interferon with endotoxin-induced interferon indicates that they are different (13).

Finally, the interferon-like molecule found in this study seems rather unique not only from the standpoint of induction but also because of its lack of neutralization by specific antibody (Table III). We are currently directing our efforts to the elicidation of these properties and the molecular events necessary for induction of this interferon. The very existence, however, of such a rapidly activated regulation of interferon production in lymphoid cells suggests an important role in lymphocyte response and immune regulation.

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