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Translation of mRNA for human lymphotoxin in microinjected *Xenopus* oocytes

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Synthesis and secretion of biologically active human lymphotoxin (LT) can be detected in *Xenopus laevis* oocytes following their inoculation with poly(A^+) RNA from human stimulated peripheral blood lymphocytes, but not in oocytes inoculated with RNA from unstimulated lymphocytes or from fibroblastoid cells. In size-fractionating mRNA of stimulated lymphocytes most LT activity is found to be coded for by RNA with an approximate sedimentation value of 19 S.

Lymphotoxin *mRNA translation* *Interferon-γ* *Lymphokine* *Lymphocyte stimulation* *Oocyte*

1. INTRODUCTION

Upon stimulation of T cells by agents such as antigens, lectins or allogeneic cells, these cells secrete proteins which can exert cytotoxic effects (review [1–3]). Similar proteins are also secreted spontaneously by cells of certain continuous B lymphocyte lines [4,5]. Our knowledge of the mechanisms by which these so-called lymphotoxins (LTs) are formed is quite limited. There is fragmentary information on the effect of various regulatory agents on the formation of LTs. Thus, interferon (IFN) and certain other lymphokines were found to enhance the production of LT in cultures of T lymphocytes ([6], unpublished), and the tumor-promoting agent phorbol-myristate-acetate increases LT secretion by cells of B lymphocyte lines (unpublished), while prostaglandin E and corticosteroids have a marked inhibitory effect on the production of LT [6,7]. However, there is no information on the nature of the intracellular events underlying the regulation of LT formation by these various agents. To approach the analysis of those mechanisms we found it necessary to define a technique for detection and quantitation of the mRNA which codes for LT. We report here that mRNA from human peripheral blood lymphocytes, with an approximate sedimentation value of 19 S, is translated in microinjected *Xenopus* oocytes to biologically active LT and that by this translation technique the level of LT mRNA is found to be significantly increased in lymphocytes consequently to their stimulation.

Abbreviations: CHI, cycloheximide; IFN, interferon; LT, lymphotoxin

2. MATERIALS AND METHODS

2.1. *Stimulation of lymphocytes*

Human peripheral blood lymphocytes, isolated on Ficoll-Hypaque from buffy coats of freshly donated blood were used at a concentration of 10^7 cells/ml in MEM alpha medium (Gibco, NY). Prior to stimulation the lymphocytes were incubated for 12 h in the presence of a lymphokine preparation (produced as described below), since we recently found that the effectiveness of LT production was dramatically increased by such pretreatment (in preparation). Phytohemagglutinin-P (5 μg/ml) (Difco, Detroit) was then added, and the lymphocytes were further incubated for 5 h and then extracted for isolation of their mRNA. The lymphokine preparations, applied in the preincubation period, were produced by incubating peripheral blood lymphocytes with con-

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canavalin A (20 μg/ml; Miles Yeda, Israel) for 24 h; they were partially purified by adsorption to controlled pore glass (PG-350-200, Sigma, St. Louis) and desorption in 0.5 M tetramethyl ammonium chloride, then subjected to acidification to pH 2.0 followed by neutralization and applied on lymphocytes at a concentration of 0.2 μg/ml.

2.2. *Extraction and purification of RNA*

For extraction of their mRNA, cells (freshly isolated lymphocytes, lymphocytes stimulated as described above or SV-80 cells) were rinsed twice with cold phosphate-buffered saline and then solubilized in guanidine thiocyanate and their RNA sedimented through a cushion of CsCl solution, with minor modifications of a procedure described in [8]: the cells were suspended in a solution containing 4 M guanidine thiocyanate, 0.1 M Tris–Cl (pH 7.4) and 0.1 M β-mercaptoethanol, and homogenized with an 'ultra turrax' for 10 s. CsCl (1 g/2.5 ml) was then added and the homogenate was applied on a 5 ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) and spun in an SW27 Beckman rotor at 26000 rpm for 20 h. The RNA pellet was suspended in a solution containing 0.1 M sodium acetate and 0.1% SDS, extracted consecutively with phenol and chloroform and with chloroform alone and was then precipitated by adding sodium acetate (pH 5.0) to 0.2 M and 2.5 vols ethanol. Poly(A^+) RNA was isolated on oligo(dT)-cellulose columns [8].

2.3. *Fractionation of poly(A^+) RNA by electrophoresis through agarose gel in the presence of methylmercuric hydroxide and by sedimentation through a sucrose gradient*

Fractionation of poly(A^+) RNA by electrophoresis was carried out according to the procedure of [9] as described in [8]. Following electrophoresis, the gel was sliced into 2 mm slices. Each slice was crushed with a spatula and suspended in 1.0 ml of a solution containing 0.5 M NaCl, 50 mM Tris–Cl pH 7.4, 5 mM EDTA, 10 mM dithiothreitol and 20 μg *E. coli* tRNA. It was then homogenized by passing twice through a 21 gauge needle. SDS was added to 0.02%, and the solution was extracted, once with phenol and chloroform and twice with chloroform alone. The RNA was then precipitated by adding sodium acetate (pH 5.0) to 0.2 M and ethanol to 70%. After rinsing the RNA pellets with 80% ethanol each pellet was solubilized in 3 μl water. For fractionation on a sucrose gradient a sample of 50 μg poly(A^+) RNA was denatured by heating for 5 min at 65°C in the presence of 0.5% SDS, and then applied on a gradient of 5–30% (w/v) sucrose in 0.5% SDS, 10 mM Tris–Cl (pH 7.4) and spun for 9 h at 40000 rpm in an SW41 Beckman rotor. Fractions of 0.4 ml were then collected and following the addition of *E. coli* tRNA (20 μg/fraction) the RNA of each fraction was precipitated in sodium acetate pH 5.0 and ethanol, rinsed with 80% ethanol and solubilized in water as described above.

2.4. *Translation of the RNA in microinjected oocytes*

X. laevis oocytes were microinjected in groups of 10, each oocyte receiving 10 nl of a solution of poly(A^+) RNA in distilled water (1 μg/ml) or water alone. Each group of oocytes was then incubated for 24 h at 22°C in 100 μl of Barth's solution supplemented with BSA (0.1 mg/ml) and the following protease inhibitors: leupeptin (2 μg/ml), pepstatin (4 μg/ml), bacitracin (2 mg/ml), aprotinin (0.4 U/ml), and EGTA (2 mg/ml). The inclusion of these compounds, for the purpose of inhibiting proteolytic enzymes which are released by the oocytes [10], increased significantly the yield of LT activity in the oocyte incubation medium. Following incubation, the oocytes were either homogenized in their incubation media or separated from the incubation medium and homogenized in a fresh sample of 100 μl Barth's medium. Homogenization was done by crushing the oocytes with a small plastic rod and then pressing them several times through a micropipet. The oocyte homogenates and the oocyte incubation media were diluted 1:1 with Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, and spun in a microcentrifuge for 2 min at 10000 rpm. Fat-free fluid was collected from the spun samples and assayed immediately for LT and IFN activities. Oocytes from different frogs differed markedly in the efficiency of translation of LT (and of IFN-γ) mRNAs. On the other hand, the variability of LT yield from one sample of oocytes to another, when injecting RNA to oocytes of the same frog, was just in the range of 30% of the average. Several frogs were therefore screened for optimal translational activity, and in each ex-

periment oocytes taken from a single frog were used.

2.5. *Quantitation of LT and of IFN activities*

LT activity was quantitated as in [11]. The samples tested were diluted serially and applied at the various dilutions, in duplicates, to confluent cultures of human SV-80 cells [12], grown in 9-mm microwells. Except where otherwise indicated, CHI at 50 μg/ml was added to the cells simultaneously with LT. Following incubation with the samples tested, for the indicated period, viability of the cells was determined by measuring the uptake of neutral red [11]. The extent of cell death is presented as a percentage, comparative to the amount of neutral red taken up by cells incubated with CHI alone. In the absence of LT and in the presence of CHI alone, SV-80 cells remain viable for at least 24 h. One unit of LT is defined as the concentration which, when applied on SV-80 cells simultaneously with CHI (50 μg/ml), kills 50% of the cells during 12 h of incubation.

IFN activity was determined on human WISH cells [13] by measuring the reduction in the cytopathic effect of vesicular stomatitis virus. One unit of IFN activity was defined as the concentration of IFN at which 50% of the infected cells were protected. Viral cytopathy was quantitated similarly to the quantitation of LT cytotoxicity by measuring the reduction in uptake of neutral red.

2.6. *Materials*

X. laevis females were purchased from the South African snake farm (Fish Hoek, South Africa). Methylmercuric hydroxide (1 M in water) was obtained from Alfa Division, Ventron (Danvers, MA). LT was produced and partially purified as in [11]. Goat antiserum against human lymphokines, raised by repeated immunization with crude preparations of IFN-γ, was a gift from Dr E. Falcoff, Institut Curie, Paris, France. One ml of that serum could neutralize 350 units LT.

3. RESULTS AND DISCUSSION

Cytotoxic activity could be detected in *Xenopus* oocytes 24 h after their inoculation with poly(A^+) RNA from human, stimulated, peripheral blood lymphocytes. As shown in fig.1, cytotoxicity was exerted by homogenates of these oocytes as well as

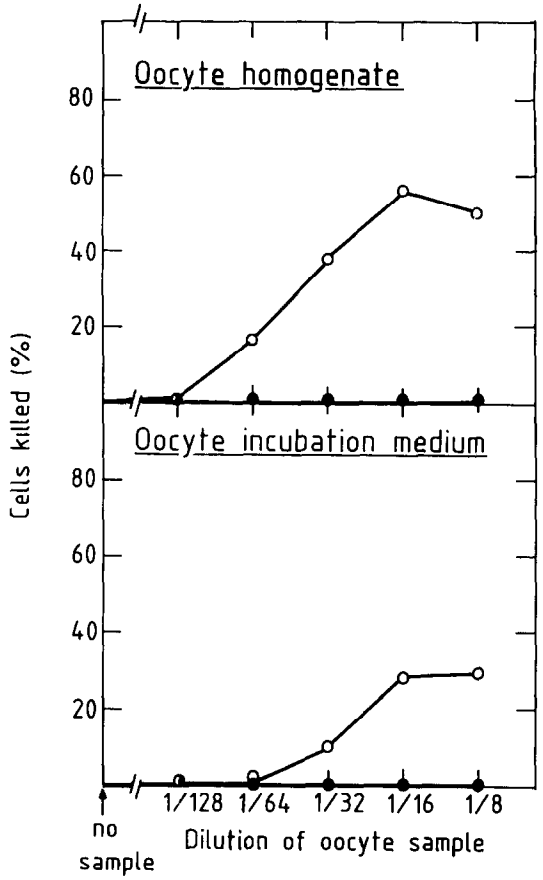

Fig.1. Cytotoxic activity in oocytes injected with mRNA of stimulated lymphocytes (○), compared with oocytes injected with mRNA of SV-80 cells (●). Each oocyte was microinjected with 100 nl containing 100 ng RNA, and incubated in groups of 10, for 24 h in 100 μl Barth's solution. Samples of the oocyte incubation media and of homogenates of the oocytes (100 μl homogenates prepared from each group of 10 oocytes) were serially diluted and applied, at the indicated dilution, on SV-80 cells together with CHI (50 μg/ml). 20 h later the extent of cell killing by the samples tested was determined by the neutral-red uptake assay. Other details as described in section 2.

by the oocyte-incubation medium, indicating that the compounds responsible for that activity were partly secreted by the oocytes. Human SV-80 cells (fig.1), human HeLa cells, and mouse L929 cells (not shown) were similarly vulnerable to the cytotoxicity produced in the oocytes.

The following evidence indicates that the cytotoxicity was due to synthesis of human LT, coded for by the microinjected RNA: (1) The

Table 1

The cytotoxic activity produced in oocytes injected with lymphocyte RNA can be neutralized by an antiserum against human lymphokines and is dependent on sensitization of the target SV-80 cells with CHI. RNA from unstimulated lymphocytes or from fibroblastoid cells do not code for cytotoxic activity

Expt.	Samples injected into oocytes	Conditions of assay		LT effect (% cells killed)
		CHI	Neutralization with an antiserum against lymphokines	
1	water	+	–	<2
	poly(A^+) RNA from SV-80 cells	+	–	<2
	poly(A^+) RNA from stimulated lymphocytes	+	–	37
	poly(A^+) RNA from stimulated lymphocytes	+	goat antiserum against human lymphokines (1:40)	<2
	poly(A^+) RNA from stimulated lymphocytes	+	normal goat serum (1:40)	35
	poly(A^+) RNA from stimulated lymphocytes	–	–	<2
2	poly(A^+) from unstimulated lymphocytes	+	–	<2
	poly(A^+) from stimulated lymphocytes	+	–	41

24 h following microinjection, the oocytes were homogenized in their incubation medium and applied, at a dilution of 1:16, on SV-80 cells in the presence or absence of CHI (50 μg/ml). To neutralize the cytotoxic activity with antiserum against lymphokines, the oocyte homogenate was incubated with goat antiserum against human lymphokines or, for comparison, with normal goat serum, both at a dilution of 1:40, for 1 h at 4°C. The extent of cell killing was determined 20 h following application of the oocyte homogenate. Each of the two experiments presented in this table as well as the two presented in table 2 were carried out twice with qualitatively the same results

cytotoxic activity could be fully neutralized by an antibody raised against human lymphokine preparations (table 1, expt.1). (2) No cytotoxic activity could be observed in oocytes which had not been injected with mRNA (table 1, expt.1), or in oocytes injected with mRNA from human fibroblastoid cells or from unstimulated lymphocytes (fig.1 and table 1, expts 1 and 2). (3) Similar to the cytotoxic effect of lymphocyte-produced LT [11], the cytotoxic activity produced by the microinjected oocytes could be exerted on the SV-80 cells only when they were sensitized by metabolic blockers such as cycloheximide (CHI) (table 1, expt.1). (4) In the absence of CHI, LT preparations not only failed to kill the SV-80 cells but rather induced, in these and in various other cells, resistance to their own cytotoxicity, as reflected in decreased vulnerability to a subsequent incubation with LT in the presence of CHI [11]. As shown in table 2, lymphocyte-produced LT cytotoxicity and the cytotoxicity produced in the oocytes behaved identically with respect to the characteristics described above: lymphocyte-produced LT preparations induced, in the absence of CHI, resistance to the cytotoxic activity produced by the oocytes (table 2, expt.1), and conversely, treating SV-80 cells in the absence of CHI with incubation medium of oocytes which had been inoculated with lymphocyte RNA (but not of oocytes inoculated with RNA from fibroblasts or water) decreased their vulnerability to the cytotoxic effect of lymphocyte-produced LT (table 2, expt.2).

Table 2

In the absence of CHI, LT preparations, produced by stimulated lymphocytes and by oocytes injected with lymphocyte mRNA have mutual protective effects against their cytotoxicity

Expt.		Pretreatment for 4 h in the absence of CHI		Treatment for 12 h in the presence of CHI	
		I Sample applied	II LT concentration in pretreatment	III Sample applied	IV LT effect (% cells killed)
1	Protection against oocyte-produced cytotoxicity by lymphocyte-produced LT	–	0	oocyte-produced LT (0.3 U/ml)	24
		lymphocyte-produced LT	250 U/ml	oocyte-produced LT (0.3 U/ml)	3
2	Protection against lymphocyte-produced LT by oocyte-produced LT preparation	Incubation medium of oocytes which were:			
		– microinjected with water	0	lymphocyte-produced LT (0.45 U/ml)	34
		– microinjected with SV-80 RNA	0	lymphocyte-produced LT (0.45 U/ml)	35
		– microinjected with RNA from stimulated lymphocytes	0.4 U/ml	lymphocyte-produced LT (0.45 U/ml)	18

Confluent cultures of SV-80 cells were incubated for 4 h in the absence of CHI, with the samples indicated in column I, then rinsed twice and incubated for 12 h, in the presence of CHI (50 μg/ml), with the samples indicated in column III. The extent of cell killing by the end of the second incubation, as quantitated by the neutral-red uptake assay is given under column IV. 'Oocyte-produced LT' was prepared by injecting oocytes with mRNA of stimulated lymphocytes, incubating them for 24 h and then homogenizing them in their incubation medium. It was applied on the SV-80 cells at a dilution of 1:16

To determine the molecular size of the mRNA which codes for LT in oocytes, poly(A^+) RNA of stimulated lymphocytes, denatured with methylmercuric hydroxide, was fractionated by electrophoresis through an agarose gel and the RNA fractions were assayed for translation to LT in microinjected oocytes. To check the resolving power of this procedure the fractionation pattern of mRNA for another lymphokine: interferon-γ was determined simultaneously by measuring also the production of antiviral activity in the microinjected oocytes. Consistent with observations of others [14–18], we found that the mRNA for IFN-γ migrated upon electrophoresis as a single species and that its sedimentation value was about 16.5 S. On the other hand, most mRNA coding for LT migrated with an approximate sedimentation value of 19 S, which could be calculated to correspond to a size of about 2300 nucleotide residues. Very low but reproducible cytotoxicity was also observed in oocytes inoculated with mRNA of about 17 S (fig.2). A somewhat lower sedimentation value for the mRNAs of LT (15 S) and of IFN-γ (12 S) were observed in fractionating RNA of stimulated lymphocytes by sedimentation through a sucrose gradient (as described in section 2). That difference between the two fractionation procedures may be related to the retainment of some secondary structure of the RNAs upon sedimentation through sucrose in the absence of an effective denaturing agent.

The presence of mRNA which can code for LTs

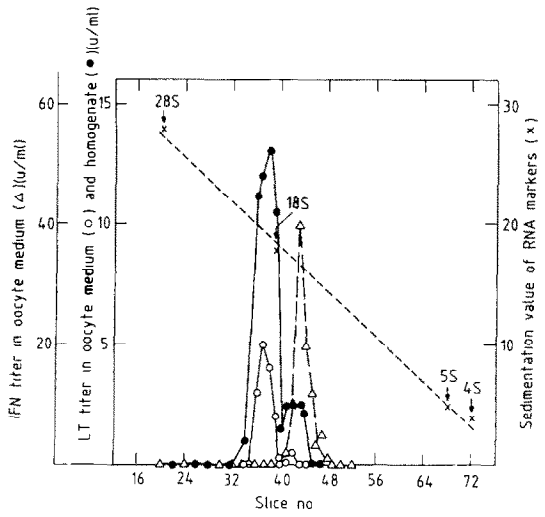

Fig.2. Electrophoresis of mRNA for LT and IFN-γ through agarose–methylmercuric hydroxide gel. A (50 μg) sample of poly(A^+) RNA from stimulated human peripheral blood lymphocytes, was mixed with radioactive RNA markers (^{32}P-labeled cytoplasmic RNA of Vero cells) and subjected to electrophoresis through a horizontal slab gel of 1.2% agarose in the presence of methylmercuric hydroxide [8,9] until the bromophenol blue marker dye migrated 18 cm. The gel was then sliced into 2 mm slices which were counted by Cerenkov counting to locate the position of the radioactive markers (×). The RNA extracted for each of the slices (see section 2) was microinjected into oocytes and the level of cytotoxic activity in the oocyte homogenates (●), as well as cytotoxic activity (○) and IFN (Δ) in the oocyte incubation media were determined at 24 h following microinjection as described in section 2. LT and IFN levels are expressed in terms of their titer in the oocyte incubation media and in the oocyte homogenates.

in lymphocytes stimulated to produce these proteins and the absence of such translationally active mRNA in nonstimulated lymphocytes (table 1, expt.2) indicate that LT formation is regulated at the level of gene expression. The oocyte translation technique can be a valuable aid in further study of this regulation and in cloning the LT genes. It can serve as an assay to determine the extent of enrichment of LT mRNA in RNA preparations used for construction of the cDNA library from which the LT gene may be cloned and can be used to identify such clones by determining whether mRNA which they 'fish out' can be translated to LT in microinjected oocytes. Finally, translation of human LT mRNA to biologically active molecules, in as large a cell as the *Xenopus* oocyte, may become a useful model system for ultrastructural study of synthesis, intracellular transport and secretion of this protein.

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