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Partial characterization of γ (immune) interferon mRNA extracted from human lymphocytes

(12-*O*-tetradecanoylphorbol 13-acetate/phytohemagglutinin/*Xenopus laevis* oocytes/lymphokines/lymphocyte cultures)

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ABSTRACT **γ (immune) interferon (IFN-γ) was induced in cultures of fresh human lymphocytes by combined treatment with a phorbol ester (12-*O*-tetradecanoylphorbol 13-acetate, TPA) and the T cell mitogen phytohemagglutinin (PHA). Compared to the IFN-γ yields obtained with PHA induction alone, the inclusion of TPA caused a significant enhancement of IFN-γ production. Poly(A)-containing mRNA was isolated from mononuclear cells induced with TPA and PHA. Injected into *Xenopus laevis* oocytes, this mRNA preparation gave rise to IFN activity with characteristic properties of human IFN-γ. Sucrose density gradient centrifugation analysis showed that IFN-γ mRNA sedimented at 15 S, suggesting that it contains a total of about 1400 nucleotides.**

As a result of recent progress in the study of various interferons (IFNs), all known IFNs can be grouped into three major classes designated IFN-α ("leukocyte"), IFN-β ("fibroblast"), and IFN-γ ("immune"). Identification of the three major IFN classes is based primarily on antigenic differences (1). Additional distinguishing properties include differences in various physicochemical and biological characteristics (2).

Several earlier studies showed that mRNA for IFN-α and IFN-β can be extracted from cells actively producing IFN. Such IFN mRNA preparations can direct synthesis of biologically active IFN molecules with corresponding specificity when introduced into heterologous animal cells (3), suitable cell-free systems (4–6), or *Xenopus laevis* oocytes (6–8). Identification of human IFN-α and IFN-β mRNAs on the basis of their ability to direct the synthesis of homologous, biologically active IFN molecules on injection in *X. laevis* oocytes made possible the preparation of cDNAs and their cloning in bacteria (9, 10). Comparison of the first available IFN-α and IFN-β cDNA sequences indicated only 29% homology between the two IFNs at the amino acid level (11). Recent analysis of human chromosomal gene bank DNA revealed the existence of at least eight related but distinct IFN-α sequences, differing from each other in about 10–30% of their predicted amino acid residues (12). In contrast, only one subclass of IFN-β mRNA has been tentatively identified in addition to the IFN-β cDNA clone originally isolated (13).

Studies of isolated IFN-α and IFN-β DNA clones and of the corresponding IFN polypeptides produced in bacteria have already provided a wealth of information about the structure and biological properties of these IFNs. In addition, cloning of these IFN genes in *Escherichia coli* promises to facilitate the production of large quantities of biologically active IFN.

In contrast, much less information is available about IFN-γ. The latter IFN is usually produced in cultures of lymphocytes exposed to various mitogenic stimuli—e.g., specific antigens or lectins. Recently we described an efficient method of production and partial purification of human IFN-γ (14). In this paper we report on the preparation of biologically active human IFN-γ mRNA. It is hoped that the preparation of IFN-γ mRNA will allow the synthesis and cloning of IFN-γ cDNA.

MATERIALS AND METHODS

Materials. Ficoll/Hypaque was obtained from Pharmacia. Eagle's minimal essential medium and RPMI 1640 medium were obtained from GIBCO. Purified phytohemagglutinin (PHA) was from Burroughs Wellcome (Research Triangle Park, NC) or from P-L Biochemicals. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was from Consolidated Midland (Brewster, NY). Oligo(dT)-cellulose (type 7) was obtained from P-L Biochemicals. All other chemicals were from standard sources.

Production and Extraction of IFN-γ mRNA. Induction of IFN-γ in human lymphocytes by PHA in the presence of TPA was carried out as described (14). Briefly, mononuclear cells were isolated from lymphocyte-rich "platelet residues" (obtained through the courtesy of Alan Waldman from the New York Blood Center) by centrifugation at 400 × *g* for 30 min on a Ficoll/Hypaque gradient. Cultures containing 6×10^6 mononuclear cells per ml in serum-free RPMI 1640 medium were treated with TPA (5 ng/ml) for 2 hr; PHA was then added at the concentration of 5 μg/ml. After incubation for the appropriate time at 37°C, cells were harvested by scraping with the aid of a rubber policeman in phosphate-buffered saline. In a typical experiment, 4×10^9 cells were pelleted by centrifugation at 800 × *g* for 5 min and resuspended in about 4 ml of phosphate-buffered saline. Cells were then dispersed into 80 ml of ice-cold 10 mM Tris·HCl, pH 7.5/10 mM NaCl/1.5 mM $MgCl_2$ containing 10 mM ribonucleoside–vanadyl complexes (15) in a 150-ml Corex tube. Nonidet P-40 was added to 0.3% (vol/vol) and, after thorough mixing, nuclei were removed by centrifugation at 3000 rpm for 5 min in a Sorvall GSA rotor. The supernatant was poured off into a Corex tube that contained 4 ml of 10% $NaDodSO_4$, 4 ml of 2 M Tris·HCl at pH 9.0, and 2 ml of 0.25 M EDTA, and immediately extracted by phenol. The extraction was repeated twice and RNA was then precipitated by ethanol. Oligo(dT)-cellulose column chromatography was carried out as described (9).

Fractionation of the mRNAs by Sucrose Gradient Centrifugation. Poly(A)-containing mRNA was heated at 70°C for 2 min and fractionated on a 5–25% linear sucrose gradient in 50 mM Tris·HCl, pH 7.5/0.2 M NaCl/1 mM EDTA by centrifugation at 26,000 rpm for 19 hr at 4°C in a Hitachi PRS 40 Ti rotor. Ribosomal RNA was sedimented in a parallel tube as size marker. Twenty-one fractions were collected and RNA in each fraction was precipitated by ethanol.

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Abbreviations: IFN, interferon; PHA, phytohemagglutinin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Translation of IFN-γ mRNA in *X. laevis* Oocytes. Microinjection of mRNA into *X. laevis* oocytes was carried out as described (16). In the case of total poly(A)-RNA, RNA was dissolved in 10 mM Tris·HCl, pH 7.5/88 mM NaCl (oocyte injection buffer) at the concentration of 0.5 or 1 mg/ml. Groups of 10 oocytes were incubated in 0.1 ml of Barth's medium for 24 hr at 24°C. The oocytes were then homogenized and the extract was assayed for IFN activity.

Assay of IFN-γ. IFN activity was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus or encephalomyocarditis virus in human GM-258 cells as described (14). Because no reference standard for human IFN-γ is available, all results were expressed in "laboratory units" (14).

RESULTS

Effect of TPA on the Yield of PHA-Induced IFN-γ and Its mRNA. Table 1 shows IFN yields obtained from cultures of mononuclear cells induced with PHA alone or by the combined treatment with TPA and PHA. IFN yields obtained after stimulation with PHA were quite variable, with cells from some donors producing over 1000 units/ml, but with some other units producing much lower or undetectable amounts of IFN. In contrast, cultures induced by the combined treatment with TPA and PHA produced relatively more uniform IFN yields, due to a marked enhancement of IFN production in cells that responded poorly to induction with PHA in the absence of TPA. Culture volume did not appear to greatly affect IFN production, because similar yields were obtained in 1-ml microcultures and in bulk cultures of 100–120 ml. (The same concentration of 6×10^6 mononuclear cells per ml was employed in both types of cultures.) Although the kinetics of IFN production proved to be somewhat variable in the experiments shown in Table 1 as well as in other experiments not included in this table, the results showed that high IFN yields were reached by 24 hr after stimulation. Therefore, in experiments aimed at the extraction of IFN-γ mRNA, cells were harvested between 16 and 36 hr after induction. Most extractions were carried out from cells harvested at 24 hr after stimulation.

In an earlier study we demonstrated that IFN produced in cultures of mononuclear cells derived from platelet residues stimulated with TPA and PHA had the characteristics of IFN-γ (14). To ascertain the suitability of the TPA/PHA stimulation for the extraction of IFN-γ mRNA we performed the following experiment. Mononuclear cells (about 5×10^9 total) obtained by Ficoll/Hypaque centrifugation from one donor's platelet residue were divided into halves. One half of the cells was first incubated with human IFN-β (fibroblast IFN) at 500 units/ml for 2 hr and then stimulated with PHA (5 μg/ml). Such "priming" with human IFN-α or IFN-β was shown to cause enhancement of PHA-stimulated IFN-γ production (ref. 17; unpublished data). The other half of the cells was incubated for 2 hr with TPA and then stimulated with PHA as described in *Materials and Methods*. At 16 hr after the addition of PHA, cells were harvested from both groups of cultures, and mRNA was isolated by phenol extraction and oligo(dT)-cellulose column chromatography. Activity of the mRNA preparations isolated from the two groups of cultures was compared by injecting them at the same concentrations into *X. laevis* oocytes and assaying oocyte extracts for interferon activity. The mRNA extracted from PHA-stimulated cells primed with IFN-β produced no detectable IFN activity on oocyte injection (<16 units/ml). In contrast, mRNA from TPA/PHA-induced cells produced clearly demonstrable IFN activity (maximum 196 units/ml).

Table 1. Effects of TPA on IFN-γ production in cultures of mononuclear cells derived from platelet residues

Platelet residue no.	Interferon titer*		
	Microculture†		Bulk culture‡
	PHA	TPA/PHA§	TPA/PHA§
1	80 (24)	5,120 (24)	5120 (40)
	120 (48)	1,280 (48)	
	40 (144)	2,560 (144)	
2	1280 (72)	1,920 (72)	1280 (24)
3	1280 (114)	5,120 (114)	2560 (24)
4	1920 (138)	640 (138)	2560 (24)
5	<40 (90)	3,840 (90)	2560 (24)
6	<40 (90)	20,480 (90)	5120 (24)

* Numbers in parentheses denote time of harvesting in hours after the addition of PHA.
† Volumes of 1 ml in 24-well microplates.
‡ Volumes of 100–120 ml in 150-mm-diameter Petri dishes.
§ TPA added at −2 hr.

On the basis of this preliminary experiment all subsequent mRNA extractions were done from cells induced by the combined TPA/PHA treatment.

Analysis of IFN-γ mRNA by Sucrose Density Gradient Centrifugation. RNA was extracted from TPA/PHA-induced cells derived from seven residues (representing a total of 3.4×10^{10} cells). Cells from each residue were cultured and extracted separately. A total of 40 mg of RNA was obtained and pooled. Of this RNA, 33 mg was processed by oligo(dT)-cellulose column chromatography. Elution of the oligo(dT)-cellulose column yielded 900 μg of RNA, which was subsequently fractionated by sucrose density gradient centrifugation.

The absorbance profile of the RNA after fractionation by sucrose density gradient centrifugation (Fig. 1) indicated that about half of the RNA yield represented contaminating ribosomal RNA, not eliminated by passing through the oligo(dT)-cellulose column. The bulk of IFN mRNA activity was recovered in fraction 12, which contained an estimated 16 μg of RNA. The sedimentation coefficient of this RNA was calculated to be approximately 15 S. Because of the proximity to 18S ribosomal RNA, the fraction containing the bulk of IFN mRNA is likely to be slightly contaminated with ribosomal RNA.

Fractionation by sucrose density gradient centrifugation resulted in some enrichment of IFN mRNA activity. A small sample of the RNA obtained from the oligo(dT)-cellulose column was analyzed for IFN mRNA activity before fractionation by sucrose density gradient centrifugation. This crude mRNA preparation injected into *X. laevis* oocytes at a concentration of 0.5 mg/ml produced an IFN yield of 80 units/ml. By comparison, RNA from fraction 12 of the sucrose gradient, injected into oocytes at a concentration of 0.32 mg/ml, yielded 320 units/ml.

Antigenic Properties of IFN Produced in Oocytes. One of the important characteristics of IFN-γ is its lack of neutralization by antisera prepared against human IFN-α or IFN-β. To ascertain that IFN synthesized in oocytes injected with RNA from TPA/PHA-induced mononuclear cells is indeed IFN-γ, we examined its affinity to a serum with high neutralizing activity against IFN-α and β (Table 2). This oocyte-derived interferon preparation was not neutralized, indicating that the activity was due to neither IFN-α nor IFN-β and, therefore, it was likely to be IFN-γ. A control preparation of oocyte-derived IFN-β produced in the same batch of oocytes by injection with mRNA extracted from poly(I)·poly(C)-induced human fibroblasts (16) was neutralized by this antiserum. Control human IFN-α and IFN-β preparations (reference standards G-023-901-527 and G-023-902-527, respectively) were both com-

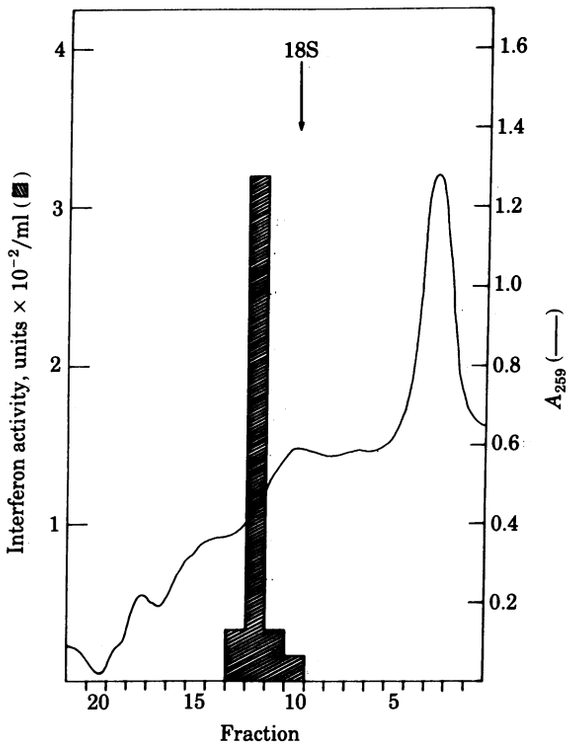

FIG. 1. Sucrose density gradient centrifugation analysis of IFN-γ mRNA. About 900 μg of RNA eluted from the oligo(dT)-cellulose column was fractionated by sucrose density gradient centrifugation. RNA in each of the resulting fractions was precipitated with ethanol and dissolved in 40 μl of H_2O per fraction. Four microliters from each fraction was mixed with 1 μl of 5× concentrated oocyte injection buffer. This material was tested for activity by injection into groups of 10 oocytes. IFN activity found in extracts of oocytes prepared 24 hr after injection is shown in the graph. Absorbance readings are of endogenous RNA present in the preparation eluted from oligo(dT)-cellulose.

pletely neutralized, but a control preparation of TPA/PHA-induced IFN-γ produced in this laboratory was not.

DISCUSSION

The phorbol ester TPA, a known tumor-promoting agent (18), induces many different biological effects in various cells in culture or in the animal. Among other activities, TPA was shown to exert a comitogenic effect in lymphocytes (19), to stimulate production of a lymphokine in a macrophage cell line (20), to induce small quantities of interferon in a lymphoblastoid cell line (21), and to enhance IFN-γ production in human lymphocytes stimulated by lectins (22). The results of this study suggest that enhancement of IFN-γ production in cells treated with TPA is accompanied by enhanced synthesis of IFN-γ mRNA. Therefore, inclusion of TPA as a coinducer along with PHA or some other suitable lectin is likely to facilitate the isolation of IFN-γ mRNA in quantities sufficient for the preparation of cDNA and its subsequent cloning.

The sedimentation coefficient of IFN-γ mRNA was calculated to be 15 S by sucrose density gradient centrifugation analysis. Although the RNA preparation was subjected to heating at 70°C for 2 min before sucrose gradient centrifugation, possible aggregation of RNA molecules cannot be completely ruled out. Therefore, the calculated value of 15 S for human IFN-γ mRNA has to be considered as somewhat preliminary. However, the fact that a single sharp peak of mRNA activity was obtained speaks against extensive aggregation. Under similar

Table 2. Lack of neutralization of oocyte-derived IFN-γ by antiserum to IFN-(α + β)

IFN preparation	IFN titer: No antibody	IFN titer: With anti-IFN-(α + β)*
Oocyte-derived IFN-γ†	384	512
Oocyte-derived IFN-β	512	<4
Control IFN-α	1024	<4
Control IFN-β	128	<4
Control IFN-γ	512	768
Control oocyte extract‡	<4	<4

* Antiserum produced by immunization of a rabbit with human IFN-α, containing neutralizing antibodies against IFN-α and IFN-β (but not IFN-γ). IFN preparations were incubated with an excess of this antiserum or with a control serum (no antibody) for 1 hr at 37°C and then assayed for IFN activity.

† mRNA isolated from cultured mononuclear cells stimulated with TPA/PHA was purified by sucrose gradient centrifugation. This material was injected into *X. laevis* oocytes and an IFN extract was prepared.

‡ From oocytes injected with buffer.

conditions of analysis the sedimentation coefficient of human IFN-α and IFN-β mRNAs was shown to be about 12 S (9, 10, 16, 23).

On the basis of earlier analysis for other mRNA species, 15S mRNA would be about 1400 nucleotides long, which could be translated into a polypeptide up to about 45,000–48,000 in molecular weight (24). The molecular weight of human IFN-γ was recently estimated to be 58,000 ± 3000 by gel filtration (14). The difference between the value based on mRNA size and the apparent size of the IFN-γ molecule itself could be due to carbohydrate. Indirect evidence suggests that native human IFN-γ is a glycoprotein (14). Alternatively, the 58,000 molecular weight form could be an aggregate or oligomer with the true molecular weight of IFN-γ being significantly smaller.

The fact that IFN-γ is antigenically different from other human IFN species and that—as demonstrated here—a distinct mRNA exists for IFN-γ strongly suggests that IFN-γ is coded for by structural gene(s) distinct from the IFN-α and IFN-β genes. Cloning of IFN-γ cDNA in *E. coli* and its subsequent sequence analysis would allow a direct comparison of the structural features of IFN-γ with the other IFN species.

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