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MESSENGER RNA OF HUMAN IMMUNE INTERFERON : ISOLATION AND PARTIAL CHARACTERIZATION

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Suspension cultures of total and lymphocyte-enriched peripheral white blood cells were used as a source for the preparation of mRNA for human γ-interferon. The cells were lysed and total RNA was extracted with the phenol method. A poly(A)-rich RNA fraction was isolated by affinity chromatography on oligo(dT)-cellulose and further purified on a preparative sucrose gradient. The RNA preparations were translated by oocytes into (a) protein(s) that showed biological activity in the interferon-assay. On sucrose gradient centrifugation the translatable fractions of the RNA migrated with a sedimentation coefficient of approximately 15 S, a value compatible with the molecular weight of human γ-interferon. The translation product was further characterized by serological cross reactivity with purified γ-interferon in neutralization reactions.

INTRODUCTION

Human interferon-γ (HuIFN-γ) is the term (1) used to designate the main active component of human immune interferon (Im-IFN) (2), a lymphokine preparation that can be obtained by stimulation of human lymphocyte cultures with a variety of mitogens (3). HuIFN-γ is a protein, probably glycosylated, with a molecular weight estimated by gel filtration at 45,000 (4,5) to 58,000 daltons (6). Im-IFN induced by Concanavalin A (ConA) was shown to contain not only HuIFN-γ but also trace amounts of HuIFN-α and HuIFN-β (7), the main components of leukocyte and fibroblast IFN preparations, respectively. The HuIFN-β present in ConA-induced Im-IFN was found to be serologically related to classical HuIFN-β (from fibroblasts) but different from it by certain physicochemical and biological criteria. Furthermore, HuIFN-β and HuIFN-γ showed partial cross-reactivity in neutralization reactions with their respective antisera (8), suggesting the possibility that there are rather extensive areas of homology in their molecular structure. The isolation and characterization of the mRNA for HuIFN-γ may directly or indirectly help to clarify the relationship between these different IFNs. Taniguchi et al. (9) and Wallace et al. (10) have already succeeded in isolating and partially characterizing IFN-specific mRNAs from mitogen-induced lymphocytes. In the present study we confirm and extend their findings.

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MATERIALS AND METHODS

Reagents

12-O-Tetradecanoylphorbol-13-acetate (TPA, Sigma Chemical Cy.), purified phytohaemagglutinin (PHA, Wellcome Reagents) and A grade concanavalin A (ConA, Calbiochem) were used as interferon inducers. Ribonucleoside-vanadyl complex (RVC) was prepared according to Berger et al. (11) or purchased from Bethesda Research Laboratories.

Antiserum neutralizing HuIFN-β (titer of 1/50,000 against 10 U/ml of Hu-IFN-β) but not HuIFN-α was obtained by immunization of a goat with HuIFN-β (specific activity of 10^6 units/mg protein). Antiserum neutralizing HuIFN-γ (titer of 1/31,200 against 10 U/ml of HuIFN-γ) was prepared by immunizing a rabbit with the 45,000 dalton fraction of ConA-induced HuIFN-γ (specific activity $10^{6.5}$ U/mg protein).

Cells and IFN induction

Human lymphocytes were obtained either from pooled buffy coats or from selected cell fractions after plateletpheresis (both through the courtesy of the Blood Transfusion Service of the Belgian Red Cross at Leuven and Antwerp).

The pooled buffy coats were treated with NH_4Cl as described (12) for the preparation of leukocyte IFN; cell suspensions at 5 x 10^6 cells/ml in EMEM (Eagle's minimal essential medium) with spinner salts were induced in spinner vessels using ConA (10 µg/ml) as described elsewhere (5,7). Harvests of IFN preparations and cells for extraction were done at indicated time intervals.

The plateletpheresis residues were diluted with the same volume of PBS, layered on 1/2 volume of sodium metrizoate/Ficoll gradients and centrifuged at 400 x g for 40 min. The lymphocyte-enriched layer was washed 3 times with PBS (without Ca and Mg), once with RPMI-1640 medium and then suspended in RPMI-1640 buffered with Hepes and Tricine. Cells were cultured at 3 x 10^6 cells/ml in 100 ml in 175 cm^2 culture flasks and induced with ConA (10 µg/ml) or with a combination of TPA (5 ng/ml immediately after seeding) and PHA (5 µg/ml, after 3 h). Harvest of IFN and cells was done at 24 h.

Interferon mRNA extraction

The cells from suspension cultures were washed twice with cold PBS and resuspended in hypotonic buffer (30 ml/10^9 cells, 10 mM NaCl, 3 mM magnesium-acetate, 20 mM Tris-HCl, pH 7.4) containing 10 mM of RVC. After swelling for 10 min at 4°C cells were lysed with 1/3 volume hypotonic buffer supplemented with 5 % w/v sucrose and 1.2 % w/v Triton N101. After homogenization with a tightly fitting tissue grinder nuclei and cell debris were removed by centrifugation for 4 min at 2000 x g. Sodium dodecyl sulphate (SDS) was added to a final concentration of 1 % w/v and RNA was extracted as described elsewhere (11).

Stationary cell cultures were decanted and leaked out. Then 10 ml of hypotonic buffer with RCV at 10 mM was added for 10 min. Detachment of the lysed cell layer was obtained by gently shaking in 0.3 % v/v of Nonidet P-40. After centrifugation for 5 min at 3000 x g the cytoplasmic extract was collected in 1/10 volume of buffer containing 1 % w/v SDS, 0.05 M EDTA and 0.8 M Tris-HCl, pH 9.0. Total RNA was prepared by extracting 2 times with water-saturated phenol at room temperature. All RNA precipitations were done in 2 volumes of cold ethanol and 1/10 volume of 1 M NaCl. In some experiments RNA was purified by 3 cycles of precipitation with 2 M LiCl at 4°C for 6 - 16 h.

Oligo(dT)-cellulose chromatography and sucrose gradient centrifugation

The poly(A)-rich RNA fraction was isolated by affinity chromatography on oligo(dT)-cellulose (type 7, PL Biochemicals) essentially as described by Aviv and Leder (13). Before sucrose gradient centrifugation or oocyte injection the precipitated RNA was washed twice with 75 % ethanol and dried under vacuum.

RNA from suspension cultures was centrifuged under denaturing conditions (50 % formamide, 1 mM EDTA, 1 % w/v SDS in 0.1 M Tris-HCl, pH 7.5) on a 5 - 20 % sucrose gradient in a Beckman-rotor SW 50-1 (38,000 rpm, 15°C, 16 h). RNA from monolayer cultures was applied to 5 - 25 % sucrose gradients in 0.2 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5 and centrifuged in a MSE Superspeed 75 rotor (31,000 rpm, 4°C, 16 h).

Table 1. Extraction of mRNA from human lymphocytes stimulated for production of Im-IFN

IFN-induction			RNA-fractionation method[a]			Number of extractions done	Translation in oocytes[b] : number of positive samples (titer range, $\log_{10}$ units/ml)
Culture method	Inducer	Average yield of crude IFN after 24 h ($\log_{10}$ units/ml)	LiCl precipitation	oligo-dT chromatography	sucrose gradient centrifugation		
Suspension[c]	ConA[d]	3.4	+	-	-	8	3 (0.3 - 1.3)
		(S.E.=0.12; N=15)	+	-	+	9	5 (0.7 - 1.1)
			+	+	-	5	1 (1.6)
			+	+	+	1	1 (0.6)
			-	-	-	3	0
			-	-	+	2	0
			-	+	-	3	0
			-	+	+	23	10 (0.3 - 1.5)
Stationary[e]	ConA[d]	< 2.5	-	+	+	1	1 (> 2.8)
	PHA/TPA[f]	3.1	-	+	+	4	4 (0.5 - 2.5)
		(S.E.=0.4; N=4)					

[a] Extraction of nuclei-depleted cytoplasmic extract by phenol followed by fractionation as indicated.
[b] Injection of 50-500 ng of RNA per oocyte; incubation for 48 h; titration of oocyte-bathing medium.
[c] Spinner cultures of 5 to 50 x 10^9 cells at 5 x 10^6 cells/ml, obtained by NH_4Cl-treatment of buffy coats.
[d] 10 µg/ml added immediately after seeding; incubation for 24 h in most experiments and for shorter periods in some.
[e] Stationary cultures in polystyrene flat-bottomed flasks at 3 x 10^6 cells/ml, obtained by Ficoll/metrizoate gradient centrifugation of plateletpheresis residues.
[f] TPA (5 ng/ml) added immediately after seeding; PHA (2 µg/ml) added 3 h after seeding; incubation for 20 h.

Translation in oocytes and interferon assay

Mature oocytes were injected with 50 nl of RNA solutions and incubated in groups of 10 at 19°C for 48 h, according to the procedures originally described by Gurdon (14).

IFN was assayed in microtiter plates by inhibition of mengo-virus induced cytopathogenicity. HEp-3 cells were used for the supernatant fluid of induced cells (15) and diploid embryonic skin muscle (E_6SM strain) cells for the oocyte medium. Titration results after neutral red staining (16) were expressed in unstandardized units, i.e. the reciprocal of the end-point dilution corresponding to 50 % dye uptake.

RESULTS AND DISCUSSION

Poly-A-rich RNA was extracted from human peripheral white blood cells after in vitro induction of the interferon system with mitogens. Two types of cell populations were used : (1) unpurified peripheral white blood cells (comprising lymphocytes and monocytes, but also granulocytes and platelets) isolated from the buffy coats of regular blood donors, and cultured in large spinner flasks (2) a lymphocyte-enriched population isolated from single plasmapheresis donors and cultured in stationary polystyrene flasks. Initially preference was given to the first (buffy coat based) system because larger numbers of cells could easily be obtained at regular intervals, and because this system had proved to be suitable for the routine production of large amounts of immune IFN, with ConA as the inducer (ref. 5 and 7, and results shown in Table 1). Although poly-A-rich RNA could be obtained from these cells, this RNA had little or no potential of being translated into biologically active IFN in oocytes. Occasionally measurable active translation products were obtained (see Table 1), but these amounts were too small to allow characterization studies.

In subsequent experiments the cell population used consisted of mononuclear cells obtained by Ficoll/metrizoate centrifugation of selected cell residues of plasmapheresis donors. Since the numbers of mononuclear cells so-obtained was rather small (max. 3×10^9 cells per batch) the cells were incubated in multiple small stationary flasks rather than in single large spinner vessels. The cells were induced either with ConA or with a combination of PHA and TPA. Inclusion of TPA was reported (6) to boost the yields of immune IFN. In experiments, using small size stationary cultures, we found a 10-fold increase of the IFN-yield when PHA was used as the inducer (see Table 2). With ConA as the inducer the enhancing effect of TPA was irregular and occasionally inhibition was noted.

Table 1 shows the results of 5 experiments in which batches of stationary cultures, each containing 2 to 4×10^9 purified mononuclear cells were induced and subsequently subjected to RNA-extraction. The poly-A-rich RNA was sedimented through sucrose gradient and each fraction was tested for translation into IFN in oocytes. In each of the 5 experiments measurable translation

Table 2. Effect of TPA on IFN production by lymphocyte cultures[a] induced with mitogens

Inducer	TPA[b] present	IFN yield (log_{10} units/ml)	
		Mean	Standard error[e]
PHA[c]	-	3.08	0.324
	+	4.01	0.252
ConA[d]	-	2.79	0.307
	+	2.91	0.403

[a] Ficoll/metrizoate purified lymphocyte population from plasmapheresed donors. Stationary cultures seeded at 3 x 10^6 cells/ml, 1 ml per 2 cm^2 well.
[b] 5 ng/ml, added immediately after seeding.
[c] 2 µg/ml, added 3 h after seeding.
[d] 10 µg/ml.
[e] N = 7.

occurred. The activity in the peak fractions amounted to ∿ 50 units per µg of injected RNA. The sucrose-gradient sedimentation profile of the active mRNA is shown in Fig. 1. The peak fraction was definitely smaller than 18S, probably ∿ 15S.

In order to characterize the translation product it was reacted with a highly specific anti-HuIFN-γ serum. The results of Table 3 show that this serum completely neutralized the translation product. From previous studies on ConA-induced HuIFN-γ it was known that this IFN shows partial cross reaction

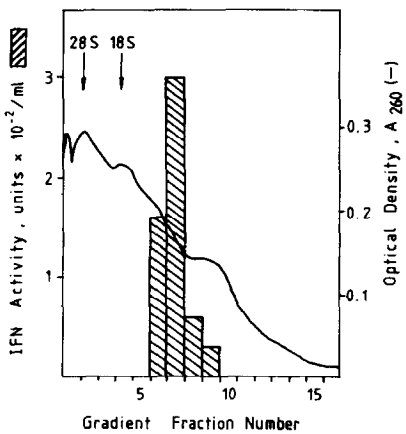

Figure 1. Sucrose-gradient sedimentation profile of poly-A-rich RNA isolated from stationary cultures of Ficoll/metrizoate purified human mononuclear blood cells, stimulated with PHA and TPA (see footnote f to Table 1).

Table 3. Serological characterization of oocyte translation product of mRNA isolated from mitogen-induced lymphocytes

Preparation tested	Reciprocal of titration end-point after reaction with :			
	No antibody	Antibody against HuIFN-γ[a]	Antibody against HuIFN-β[b]	Antibody against leukocyte IFN (HuIFN-α + -β)
Oocyte translation product,				
RNA-fraction[c] 6	50	< 4	20	n.d.
7	40	< 4	13	n.d.
8	16	< 4	< 4	n.d.
9	5	< 4	< 4	n.d.
Immune IFN				
45K-fraction	1,600	13	1,000	500
22K-fraction	100,000	6,300	< 32	40,000
Fibroblast IFN	100,000	25,000	13	4,000
Leukocyte IFN	16,000	16,000	16,000	< 10

[a] Antibody raised against 45K-fraction of ConA-induced immune IFN.
[b] Antibody raised against IFN obtained by superinduction of fibroblasts.
[c] Fraction numbers refer to Fig. 1.

with HuIFN-β. The results of Table 3 show that this was also the case with the translation product of oocytes injected with mRNA from the induced lymphocytes.

The results reported here are concordant with those of Taniguchi et al. (9) in that these authors also found a sedimentation value of 15S. In their case the translation product was characterizable by its non-neutralizability by a serum active against both HuIFN-α and -β, while in our study it was characterized in a positive way, i.e. by neutralization in reaction with a specific anti-HuIFN-γ serum. A sedimentation value of ∿ 18S was reported by Wallace et al. (10) for the mRNA of an immune IFN induced by staphylococcal enterotoxin. It cannot be ascertained at this time whether this material is truly different from the 15S mRNA in our study and in that of Taniguchi et al. (9). The latter authors estimated that a 15S mRNA could code for a peptide of 45 to 48,000 daltons, a value which is slightly less than their estimation of the molecular weight of HuIFN-γ but concordant with the estimation of 45,000 reached in our studies (5,7).

It is also possible that the 15S mRNA codes for a much smaller peptide of which the 45 or 58,000 dalton HuIFN-γ is an oligomer. A possible candidate for the monomer is a 22,000 dalton protein detectable in crude human Im-IFN and serologically related to both HuIFN-β and HuIFN-γ (7).

Molecular cloning of the 15S mRNA will be necessary to clarify the relation between HuIFN-γ and the other known IFN-types. If selection of bacterial clones is to be achieved by hybridization with mRNA, as it was done for HuIFN-α and HuIFN-β, relatively large amounts of biologically active 15S RNA will have to be prepared. It was hoped that suspension cultures of unpurified buffy coat leukocytes would be suitable for this purpose. However, for reasons that are unclear, the RNA isolated from such cultures was inactive in the oocyte translation assays. Perhaps it was too heavily contaminated with irrelevant mRNA from cells that were not involved in the immune IFN production process.

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