Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 865-871

TRANSLATION OF HUMAN IMMUNE INTERFERON MESSENGER RNA IN XENOPUS LAEVIS OOCYTES

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Received April 20, 1981

SUMMARY: Stimulation of human leukocytes in vitro with the mitogen, staphylococcal enterotoxin A, results in highly variable quantities of interferon. Of the 37 cultures tested, approximately 30% did not produce detectable γ-interferon, and about 30% synthesized significant quantities (>100 Units ml^{-1}). Messenger RNA isolated from the cells of selected donors 48 to 72 hours after mitogenic stimulation gave rise to functional γ-interferon when translated in Xenopus laevis oocytes. The mRNA coding for γ-IFN is the largest interferon mRNA so far encountered in human cells, sedimenting at 18S in formamide-sucrose density gradients. Relative to the cytoplasmic polyadenylated RNA obtained from the entire leukocyte population, the level of γ-interferon mRNA on a molecular basis was approximately ten parts per million.

INTRODUCTION

Upon stimulation with antigens or mitogens, cultured lymphocytes or mononuclear leukocytes isolated from the peripheral blood of normal donors produce human γ-interferon (also known as immune or T-IFN) (1). This class of interferon differs from both leukocyte (α) and fibroblast (β) IFNs. It is never virally induced and therefore may not function primarily as an antiviral substance in vivo. The protein, most probably a glycosylated molecule, has not been purified to homogeneity but can be distinguished from α- and β-IFN either antigenically or by its instability at pH2. Estimates of the M_r range from 40,000 to 70,000 (2) in contrast to α-IFN(15,000-21,000)(3) and β-IFN (20,000)(4). Consistent with this higher molecular weight we show here that γ-IFN mRNA is clearly unique when compared with the other, much smaller, IFN mRNAs (5-8). Its extreme scarcity among mRNAs from stimulated, unfractionated leukocyte cultures will severely hamper cloning by conventional means.

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Abbreviations used: SEA, staphylococcal enterotoxin A; IFN, interferon.

0006-291X/81/100865-07$01.00/0

MATERIALS AND METHODS

Preparation and Activation of Leukocytes Leukocytes were isolated from heparinized plateletpheresis residues (American Red Cross, Washington, D.C.) by the method of Boyum(9) and cultured as described by Berger(10) except that 10% defibrinated heterologous plasma (American Red Cross) replaced autologous plasma. After a 72h exposure to 0.2 μg ml^{-1} purified SEA (a gift of Leonard Spero, Fort Detrick, Frederick, MD), leukocyte supernatants were assayed for interferon activity. Occasionally, the proliferative activities of 72h SEA-stimulated cultures (donors #1-4, Fig. 1) were determined by measuring the uptake of (methyl-^{3}H)thymidine (52Ci $mmol^{-1}$, Amersham, Arlington Heights, IL) by 1 ml aliquots of cell culture after a 2h pulse at 1 μCi ml^{-1}.

Interferon Assay. Interferon titers of culture supernatants or oocyte bathing media were performed using GM2504 fibroblasts and encephalomyocarditis virus according to published procedures (7). In some cases (donors #15-36, Fig. 1) HSF-4 cells were substituted. Values are reported in Units ml^{-1} relative to the NIH leukocyte IFN standard unless stated otherwise.

Preparation, Fractionation and Translation of Leukocyte Cytoplasmic Poly(A)-containing mRNA. RNA was obtained from leukocyte cytoplasm using ribonucleoside-vanadyl complexes as nuclease inhibitors (11,12). Poly(A)-bearing molecules were selected by oligo(dT)-cellulose chromatography (13). Samples were fractionated in sucrose density gradients containing 70% formamide and quantified spectrophotometrically using $E^{1\%}_{260nm} = 200$. Radioactive rRNAs from BRL (Rockville, MD) were used as markers. Solutions containing approximately 1 μg μl^{-1} mRNA, except where specified to the contrary, were microinjected into mature oocytes of Xenopus laevis for translation. The aliquot size was 50 nl per oocyte. Oocyte bathing medium was subsequently assayed for IFN. These procedures are detailed elsewhere (7,14).

Acid Treatment of IFN Samples. Acid treatment was carried out by titrating the sample to pH 2 with concentrated HCl, incubating it overnight at 4° and readjusting the sample to pH 7 with concentrated NaOH. Authentic α- and β-IFNs retained virtually complete activity after being subjected to this treatment.

Neutralization of IFN with Antibodies to Human IFNs. Sheep antibody to human α-IFN (a gift of Kathryn C. Zoon, Bureau of Biologics, Bethesda, MD) at >500 neutralization Units ml^{-1} was incubated with IFN samples for 2h at 37°. Rabbit antisheep serum (Miles, Elkhart, IN) was used to precipitate immune complexes. Insoluble material was removed by centrifugation in an Eppendorf, Model 5412 centrifuge. The recovered supernatant was assayed for IFN activity.

Antibody to human β-IFN (29-33 from the National Institute of Allergy and Infectious Diseases, Bethesda, MD) at >150 neutralization Units ml^{-1} was used similarly except that Staphylococcus aureus (Pansorbin, Calbiochem-Behring, La Jolla, CA) was employed to precipitate immune complexes. The titer of the antibody to β-IFN was determined with an authentic NIH β-IFN standard.

RESULTS AND DISCUSSION

Leukocytes were isolated from plateletpheresis residues of normal donors and activated in culture with SEA. In order to supply large quantities of leukocyte mRNA, cells were prepared from pheresis residues each of which yielded 2-5 x 10^9 cells. A unit of peripheral blood, in comparison, contains approximately 20% as many leukocytes. SEA, which in related systems has been reported to be a superior γ-IFN inducer (15), stimulated the production of this substance as well as, or better than, phytohemagglutinin when leukocytes were cultured with heterologous plasma. Concanavalin A was

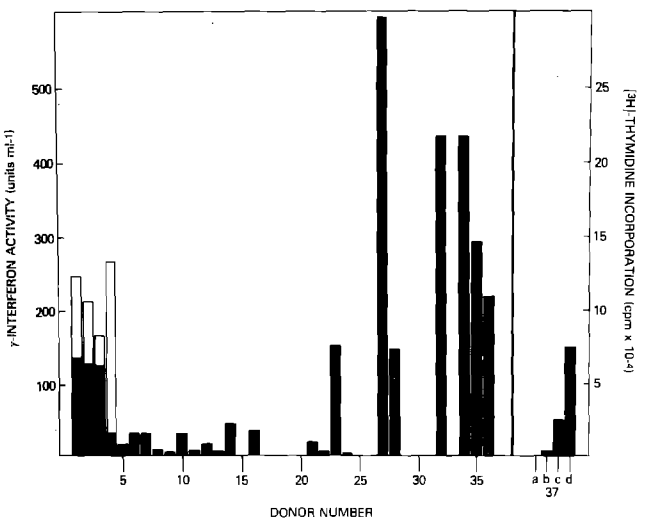

Fig. 1. Immune interferon production by cultures of human leukocytes after treatment with staphylococcal enterotoxin A (SEA) for 72h. Interferon titers in the culture fluids (black bars) and (^{3}H)thymidine incorporation (white bars) were determined using cells obtained from 37 unselected donors. Cultures which were unstimulated produced no detectable interferon; (^{3}H)thymidine incorporation was between 500-1500 cpm. Leukocytes from donor #37 (a-d) were cultured in four separate media as follows: culture 'a', MEM without serum; cultures 'b, c and d', MEM containing 10% heterologous serum obtained from three different donors. All interferon activities found in the culture supernatants were destroyed by treatment overnight at pH 2.

inferior. The ability of SEA to stimulate DNA synthesis in these cultures was unrelated to its ability to induce γ-IFN (Fig. 1, donors #1-4). Furthermore, the mitogenic activity of SEA varied with the donor, an observation characteristic of leukocytes regardless of the mitogen used for activation (16).

Using SEA as a γ-IFN inducer, we investigated cells from 37 donors. As shown in Fig. 1, 70% of the cultures produced immune interferon by 72h after induction but only 40% of these, or 30% of the total, produced sizeable quantities (>100 Units ml^{-1}). In some cases, cultures were observed for a period of 7 days. The maximum accumulation of immune IFN in capable cultures usually occurred between 3 and 6 days after induction, with measurable levels remaining through the 7th day. In contrast, cultures that had synthesized little or no IFN by day 3 did not produce it thereafter. (Data not shown.) Occasionally the defect could be traced to the serum (Fig. 1, donor #37), but removal of serum entirely, resulted in neither interferon synthesis nor mitogenic transformation. These data serve to emphasize the complexity of the human system with its heterogeneous, outbred populations.

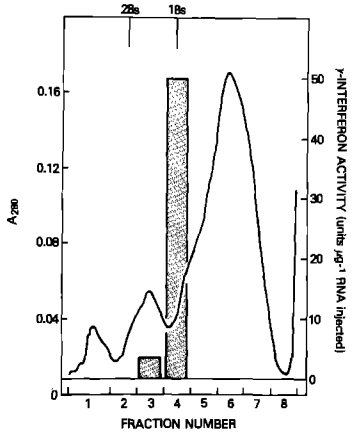

Fig. 2. Size distribution of immune IFN poly(A)-containing mRNA determined by sucrose density gradient centrifugation. Poly(A)-bearing mRNA was obtained by combining RNA from five 72h-SEA-stimulated cultures (individual donors) all of which produced more than 100 Units ml^{-1} of γ-IFN in their culture supernatants. The solid line represents A_{280nm}; the stippled bars, IFN mRNA activity.

Since maximum interferon mRNA levels immediately precede the maximum rate of interferon production in systems producing either α-(7) or β-IFN(17-19) cells were harvested and cytoplasmic RNA was isolated either at 48 or at 72h in order to obtain immune interferon mRNA. Of necessity, the preparation of RNA anticeded the results of the assay for immune interferon in the culture supernatants. Subsequently, we pooled RNA derived from selected cultures with high titers of interferon (>100 Units ml^{-1}) in order to avoid dilution of immune interferon mRNA with mRNA from nonproductive donors. Pooled polyadenylated molecules were fractionated on sucrose gradients and microinjected into Xenopus laevis oocytes (7). The secreted translation products were assayed for IFN. As illustrated in Fig. 2., the messenger RNA was heterodisperse in size. Molecules giving rise to interferon were located in a sharp peak sedimenting at 18S relative to labeled 18S and 28S rRNA markers. These mRNA molecules are clearly much larger than α-IFN mRNAs isolated either from normal leukocytes infected with Sendai virus (5) or from lymphoblastoid cells infected with Sendai(6) or Newcastle disease virus(7). They are also larger than both mRNAs which code for β-IFN (8). Thus, the 18S mRNA fraction appears to be unique in the human interferon system.

Further characterization of the interferon produced by oocytes in response to 18S mRNA from SEA-stimulated leukocytes was carried out either by treating the

Table I. Characterization of interferon produced by X.laevis oocytes injected with mRNA from 72h SEA-activated human leukocytes

Inducer and period of incubation with leukocytes	Polyadenylated mRNA 10^2 x pg cell^{-1}	Interferon mRNA activity Units μg^{-1} unfractionated mRNA
48h SEA	3	0.3
72h SEA	2	0.2
12h Newcastle Disease Virus	1	20*

Leukocyte cultures were either induced with 0.2 $\mu g\ ml^{-1}$ SEA or 25 hemagglutinin units of Newcastle disease virus, strain B-1, per 10^6 cells (7). Cytoplasmic RNA was extracted and the polyadenylated RNA prepared by one cycle of oligo(dT)-cellulose chromatography as described in Materials and Methods. Xenopus laevis oocytes were injected with the polyadenylated mRNA samples at concentrations from 2-5 $\mu g\ \mu l^{-1}$.

*IFN produced in the presence of virus was >99% α-IFN based on titrations with antibody to α-IFN.

samples with antibodies or by incubating them at pH 2. Table 1 summarizes these results. The putative immune IFN was neutralized neither by antibodies to α-IFN nor by antibodies to β-IFN. In all cases the IFN titers of the control and the antibody-treated material were the same within the 2-fold error inherent in the assay. However, the lack of precison with which IFN levels are measured makes it impossible to rule out the presence of small quantities of either α- or β-IFN using antibody neutralization data, exclusively. Oocyte IFN samples were therefore reassayed after treatment at pH 2. As indicated in Table 1, IFN activity was completely destroyed by this procedure. Since the loss of IFN activity by acid treatment is characteristic solely of γ-IFN(1), the contribution of other IFNs, if present, must be less than 0.2 Units ml^{-1}. Although none of these tests represents a positive identification, they rule out all other known interferons. The conclusion was drawn that an 18S mRNA indeed coded for immune interferon.

As part of the characterization of immune interferon mRNA, the size of the RNA was compared with that of immune interferon. Although neither the molecular weight nor the carbohydrate content of immune interferon is known with certainty, the existing information does provide guidelines. Estimates of the molecular weight ranging from 40,000-70,000 have been reported by Langford et al (2). The immune interferon mRNA we have identified is greater than 2000 nucleotides long. Its coding capacity exceeds that required for a protein moiety of 70,000 daltons. Therefore, regardless

Table II. Comparison of the levels of interferon mRNA in mitogenically and virally induced human leukocytes

Material assayed	Interferon activity Units ml^{-1}	laboratory units ml^{-1}*
Oocyte interferon†	50	1024
Acid treated oocyte interferon	<0.2	<4
Oocyte interferon + pre-immune serum (anti-α-IFN control)	25	512
Oocyte interferon + anti-α-IFN antiserum	50	1024
Oocyte interferon + Medium containing 10% fetal bovine serum (anti-β-IFN control)	36	768
Oocyte interferon + anti-β-IFN antiserum	18	384
α-interferon standard	100	2048
Medium containing 10% fetal bovine serum	<0.2	<4

*Laboratory units ml^{-1} is the reciprocal of the dilution of the test sample at which the 50% cytopathic effect was observed.
†Oocyte interferon refers to the bathing medium taken 24h after injection of oocytes with mRNA, gradient fraction number 4, from 72h SEA-stimulated human leukocytes (see Fig. 2).

of the carbohydrate content of the protein, this 18S mRNA meets the requirements for an authentic immune interferon mRNA.

The frequency of the immune interferon mRNA among bulk leukocyte mRNAs was also estimated. By measuring the interferon synthesized by oocytes injected with a known quantity of unfractionated mRNA, it was possible to deduce the interferon mRNA levels of selected mitogen-induced cultures relative to those of unselected virus-treated cultures. In the former case, the cells synthesized γ-IFN; in the latter, α-IFN, virtually exclusively. As summarized in Table 2, the immune interferon levels were approximately 1-2% of the leukocyte interferon titers after normalization. Therefore, the frequency of the SEA-stimulated immune interferon mRNA is 1-2% of that of the virally-induced leukocyte interferon mRNA. Such a comparison of the two human interferon systems depends on two assumptions: (i) the efficiency of translation of immune interferon mRNA is equivalent to that of leukocyte interferon mRNA in the oocyte system; (ii) the specific activities of α- and γ-IFNs are comparable. The relative

amounts of the two IFN mRNAs can be converted into absolute quantities provided the frequency of IFN mRNA is known for one of them. Since Weissmann and colleagues have shown tht 0.1% of the polyadenylated RNA obtained from virally-induced leukocyte cultures is α-IFN mRNA (5), it follows, given these assumptions, that the level of immune interferon mRNA in cultures from selected donors treated with staphylococcal enterotoxin A is approximately 10 parts per million on a molecular basis. Because the levels of γ-IFN mRNA activity remained constant, within experimental error, between 48 and 72h after induction, it is likely that the values stated here represent the optima for SEA-treated human leukocytes. Regardless of which cells in the population produce γ-IFN, the molecular cloning of its mRNA from these preparations using the same methods which were successful for α-IFN(5) would, on statistical grounds, require screening 100-fold more clones.

Acknowledgement: M.J.M.H. was supported by the Microbiology and Infectious Diseases Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD.

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