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Gamma-interferon induction in human lymphoblasts compared with fresh mononuclear leucocytes: earlier synthesis, rapid shut-off and enhancement of yields by metabolic inhibitors

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Summary. The mechanisms of gamma-interferon (IFN-γ) induction in fresh human peripheral blood mononuclear leucocytes (PBML) and proliferating lymphoblasts were compared. Cotreatment with mitogen (Staphylococcal enterotoxin A) and tumour promoter (mezerein) was used to induce maximum IFN-γ production and thus to study the induction process under optimum conditions. Total IFN yields were about the same from both cell types. Proliferating lymphocytes produced IFN much earlier and more transiently than fresh PBML. Experiments with actinomycin D indicated that *de novo* synthesis of RNA was required for IFN-γ production in both PBML and lymphoblasts, but that for maximal IFN-γ production, lymphoblasts required RNA synthesis for a shorter period (1 hr) after induction than did fresh PBML (greater than 15 hr). Appropriate schedules of treatment with metabolic inhibitors actually increased IFN production in lymphoblasts. This 'superinduction' could not be demonstrated for fresh PBML, implying differences in the turn-off of IFN-γ production in these two cell types. Taken together, these results indicate that IFN-γ expression is regulated differently in quiescent and activated lymphocytes.

INTRODUCTION

Gamma-interferon (IFN-γ) is produced by fresh leucocytes stimulated with mitogens (Langford *et al.*, 1981; Wheelock, 1965). Recently, it has been shown that T lymphoblasts growing *in vitro* in interleukin-2 (IL-2) containing medium also produce IFN-γ in response to mitogens (Marcucci *et al.*, 1981; Matsuyama *et al.*, 1982). The cellular mechanism of induction of IFN-γ appears to differ between fresh lymphocytes and proliferating lymphoblasts. For example accessory monocytes or macrophages are required for maximal IFN-γ production by fresh T lymphocytes (Epstein, Cline & Merigan, 1971), whilst growing lymphoblasts do not need this assistance (Marcucci *et al.*, 1981; Matsuyama *et al.*, 1982). This may be because fresh lymphocytes are resting prior to stimulation and thus must undergo differentiation (with monocyte help) before being capable of IFN-γ production; such 'differentiation' may have been already completed in activated lymphoblasts (Durum & Gershon, 1982; Larsson, Iscove & Coutinho, 1980; Smith, Gilbridge & Favata, 1980). Because T lymphoblasts have differentiated and are metabolically more active than fresh T lymphocytes, one might also expect them to differ in the mechanism of induction at the molecular level.

In this report we examine IFN-γ production by both fresh human peripheral blood mononuclear leucocytes (PBML) and growing lymphoblasts. Using metabolic inhibitors, we find differences in both the turn-on and turn-off of IFN-γ production by fresh PBML and lymphoblasts. This implies the cellular and molecular regulation of IFN-γ production differs in lymphocytes at different stages of activation.

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MATERIALS AND METHODS

Mitogens, tumour promoters and metabolic inhibitors
Staphylococcal enterotoxin A (SEA; supplied by the Food and Drugs Administration of the U.S.A., pure) and phytohaemagglutinin (PHA; Sigma, crude) were used as T cell mitogens. The tumour promoters mezerein (supplied by L. C. Services corporation, Woburn, MA, U.S.A.) and teleocidin (the kind gift of Dr. H. Fujiki, National Cancer Research Institute, Tokyo, Japan) were used to increase yields of IFN-γ (Yip *et al.*, 1981). Actinomycin D (AMD: Sigma) and cycloheximide (CHX: Sigma) were used to inhibit cellular RNA and protein synthesis.

Preparation of fresh PBML cultures
Buffy coat cells (provided by the Regional Blood Transfusion Centre, Birmingham, England) were fractionated by centrifugation of Ficoll-Hypaque (Pharmacia) as described in the Pharmacia Handbook. PBML were resuspended in RPMI-1640 medium, buffered with HEPES, and supplemented with 10% foetal calf serum.

Preparation of IL-2
Culture of fresh PBML 10^7 cells ml^{-1} (several donors' cells mixed together) were pulsed for 8 hr with 1% of a crude *Staphylococcus aureus* supernatant and 10 ng ml^{-1} mezerein. The IL-2 containing supernatant was harvested 15 hr after completing the pulse. Such supernatants efficiently support T lymphoblast growth at a 1:20 dilution.

Growth of T lymphoblasts
Cultures of fresh PBML at 10^6 ml^{-1} were stimulated with SEA (20 ng ml^{-1}). After 2 days at 37°, the cells were thoroughly washed and then resuspended at 3×10^5 cells ml^{-1} in medium supplemented with IL-2. Cultures were diluted with fresh IL-2-containing medium when they reached a density of 10^6 cells ml^{-1}.

Under these conditions, cells proliferated for up to 4 weeks and then the growth rate declined. No attempt was made to maintain the cell cultures for longer. After the first few days, more than 90% of cells in the cultures formed rosettes with sheep red blood cells and no adherent cells were present. The cultures thus consisted mainly of T lymphoblasts.

IFN induction
Fresh PBML or growing lymphoblasts were washed and resuspended in fresh medium without IL-2. SEA (20 ng ml^{-1}) with mezerein (10 ng ml^{-1}) or PHA (10 μg ml^{-1}) with teleocidin (10 ng ml^{-1}) were then added. Supernatants were harvested at intervals and stored at −70° prior to IFN assay. These experiments were carried out with cell cultures derived from individual donors (i.e. never mixed cultures).

IFN assay
This was done by the inhibition of nucleic acid synthesis method (Atkins *et al.*, 1974) using WISH (human amnion) cells challenged with Semliki Forest virus. A laboratory IFN-γ standard was included in all assays. IFN units quoted are laboratory units (there is no international IFN-γ standard).

Assay of RNA and DNA synthesis.
Cells were incubated with 5 μCi ml^{-1} 5-[^{3}H]-uridine (29 Ci/mmol) or methyl[^{3}H]-thymidine (25 Ci/mmol) for 24 hr (supplied by Amersham International). Cells were then washed once with PBS, twice with 5% cold trichloroacetic acid (TCA) and once with ethanol. The level of TCA-precipitable counts was assessed on a scintillation counter.

RESULTS

Production of IFN-γ by fresh human PBML and lymphoblasts

Growing lymphoblasts produced IFN-γ much more rapidly than did fresh PBML. Figure 1 shows the time

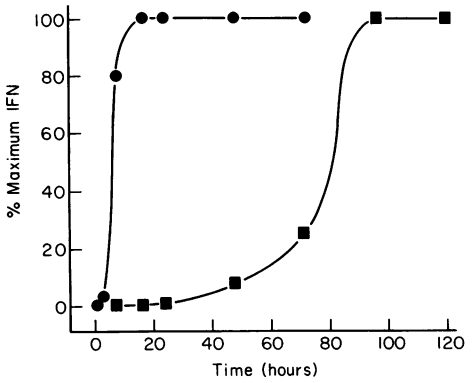

Figure 1. Cumulative yield of IFN-γ in lymphoblasts and PBML induced with SEA/mezerein. Yields are quoted as % maximum titre to facilitate direct comparison. Maximum yields in this experiment were 800 U ml^{-1} lymphoblasts and 5000 U ml for PBML, respectively. (●) Lymphoblasts, (■) PBML.

course for accumulation of IFN-γ in the supernatant of cultures of cells suspended at 5×10^5 ml^{-1}, induced with SEA/mezerein. The PBML and lymphoblasts were derived from different donors. However, in repeated experiments, we found that IFN was always produced more rapidly by growing cells than by fresh. Peak IFN titres occurred 8–16 hr after induction in lymphoblast cultures, and by 96–120 hr in fresh PBML cultures. Maximum yields of IFN from both cell types was about the same (1000–10,000 U ml^{-1}) under these conditions of induction. Other experiments were carried out in which induced cells were resuspended in fresh medium at intervals after induction. These experiments showed that more than 90% of the IFN produced by lympoblasts was secreted in the first 24 hr after induction, with very little being produced subsequently. In marked contrast, PBML produced more than 90% of the IFN *later* than 48 hr after induction, and continued secreting IFN for up to 5 days. PBML suspended at higher densities (up to 3×10^6 cells ml^{-1}) produced IFN more rapidly. However, in no experiment did PBML produce IFN as rapidly as lymphoblasts, the bulk of the IFN (90%) always being produced later than 24 hr after induction.

The IFN produced by fresh PBML and lymphoblasts was characterized as being predominantly IFN-γ by neutralization tests using antisera specific for IFN-α, -β and -γ.

The rapid decline in IFN-γ production in the lymphoblasts was not due to loss of viability or a general decline in macromolecular synthesis. High levels of RNA and DNA synthesis (as assessed by [^{3}H]-uridine and [^{3}H]-thymidine incorporation) were sustained for at least 5 days after induction; the levels were similar in PBML (Fig. 2).

It should be noted that uninduced lymphoblasts produced no IFN-γ, or at least very much less than did induced lymphoblasts. The IL-2 preparation we used contained IFN-γ, and at the IL-2 concentration used, the growth medium contained about 100 units ml^{-1} of IFN. Constitutive production of IFN levels by the cells in IL-2-containing medium of less than 100 units ml^{-1} would therefore not have been detected. However, when the lymphoblasts were resuspended in fresh medium without added IL-2, no IFN was produced in the absence of induction.

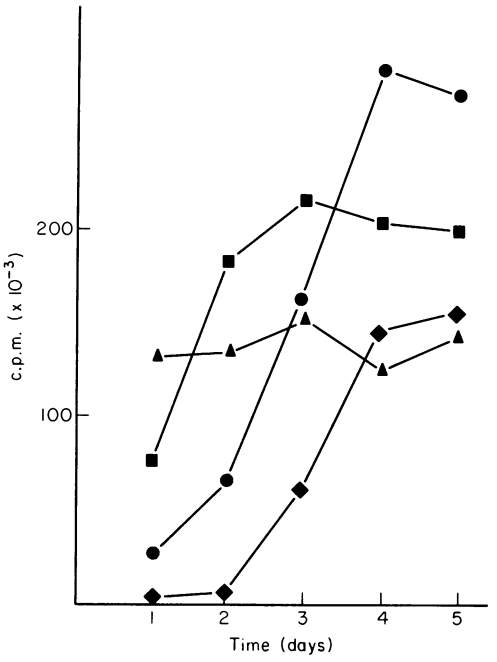

Fig. 2. Macromolecular synthesis in lymphoblasts and PBML induced with SEA/mezerein. The times indicated are the end of a 24 hr label with the appropriate isotope. The incorporation of [^{3}H]-uridine in PBML (●) and lymphoblasts (▲), and the incorporation of [^{3}H-thymidine in PBML (♦) and lymphoblasts (■) are given. (Uninduced cultures incorporated less than 15,000 c.p.m. after day 1).

Effects of AMD on IFN-γ production

The effects of AMD treatment of IFN-γ production by fresh PBML and growing T cells were found to be quite different. If fresh PBML were treated with AMD (an irreversible inhibitor of DNA-dependent RNA synthesis) prior to SEA/mezerein stimulation, little or no IFN was produced. Treatment with AMD several hours after induction substantially inhibited IFN-γ production, yields being reduced by 99% when AMD was added at 5 hr and 90% or more when AMD was added 15 hr after induction. As with fresh PBML, if growing lymphoblasts were treated with AMD prior to induction, little or no IFN-γ was produced. In contrast, if the lymphoblasts were treated with AMD as little as 1 hr after induction, there was no reduction of IFN-γ yields: there was in fact frequently an augmentation of yields (Table1). In different experiments, the augmentation was up to 10-fold, but was usually two- or three-fold.

Similar results were obtained with 3 or 10 μg/ml AMD and with inducers besides SEA/mezerein (SEA alone, or PHA/teleocidin).

Table 1. Effect of AMD on production of IFN-γ by lymphoblasts stimulated with SEA/mezerein

AMD added (10 g ml^{-1})	Time or resuspension in fresh medium (without AMD or inducer)	IFN-γ Yield at 24 hr after induction (units ml^{-1})
none	2 hr	500
1 hr	2 hr	1300
none	3 hr	500
2 hr	3 hr	1300
none	6 hr	200
5 hr	6 hr	8000

Inducers (SEA/mezerein) were added at 0 hr and AMD added at later times. One hour after addition of AMD, cells were washed and resuspended in fresh medium without inducers or AMD. Under these conditions, the control induced cultures make smaller amounts of IFN-γ compared with cultures continuously treated with inducers.

Table 2. 'Superinduction' of IFN-γ production in lymphoblasts

Inhibitor added	Time of resuspension in fresh medium (without inhibitors or inducers)	IFN-γ yield 24 hr after induction (units ml^{-1})
None		630
AMD at 4 hr		1000
Chx at 1 hr	5 hr	500
AMD at 4 hr +CHX at 1 hr		2500

Inducers (SEA/mezerein) were added at 0 hr and then lymphoblasts were treated with the inhibitors AMD and CHX as indicated. Cells were washed and resuspended in fresh medium without inducers or inhibitors.

'Superinduction' of IFN-γ in lymphoblasts

'Superinduction' is the overproduction of an induced protein in cells treated with metabolic inhibitors: it occurs in human fibroblasts production of IFN-β upon poly I-C induction. We found that 'superinduction' schedule involving treatment first with the reversible inhibitor of protein synthesis, cycloheximide (CHX), then with AMD, did indeed augment IFN-γ yields in lymphoblasts. This occurred in repeated experiments, and representative data is shown in Table 2. We have never been able to demonstrate clearly 'superinduction' in fresh PBML.

DISCUSSION

Our experiments show that the characteristics of IFN-γ induction in fresh PBML and growing lymphoblasts differ. The 'switch-on' of production of IFN-γ by growing lymphoblasts is much more rapid, and IFN production ceases earlier than in PBML. Experiments with an inhibitor of RNA synthesis (AMD) suggest that significant levels of messenger RNA (mRNA) for IFN-γ do not pre-exist in either cell type, but upon induction, lymphoblasts have a more rapid rise of steady state IFN-γ mRNA levels than fresh PBML. This has been confirmed by hybridization experiments employing a cDNA probe specific for IFN-γ (Siggens, Wilkinson & Morris, 1984, Biochem. biophys. Res. Commun., in press). The augmentation of IFN yields in lymphoblasts treated with metabolic inhibitors implies a 'shut-off' mechanism operating which requires *de novo* RNA and protein synthesis.

The reason for the more rapid production of IFN-γ, both protein and message, in the growing lymphoblasts could be their more active metabolic state compared to fresh PBML. Fresh PBML are metabolically very inert, whilst the growing T cells actively synthesize DNA, RNA and protein (Hume & Weidemann, 1980). This may be because fresh T lymphocytes require a cascade of early events before activation (Durum & Gershon, 1982; Larsson *et al.*, 1980; Smith *et al.*, 1980). These early events include the interaction of T cells with IL-1 (produced by monocytes) which allows the production of IL-2 by certain T cell subsets. This IL-2 in turn activates T cells with newly formed IL-2 receptors. IFN-γ production may be dependent on this cascade of events, since it has recently been shown that IL-2 induces IFN-γ in both human and mouse lymphocytes (Farrar, Johnson & Farrar, 1981; Kasahara *et al.*, 1983; Torres, Farrar & Johnson, 1982). In this regard, we found that IL-2 production clearly precedes IFN-γ production by fresh PBML (data not shown). The much more rapid IFN-γ production by proliferating lymphoblasts may be because these cells are already activated, bearing IL-2 receptors and growing in the presence of IL-2. We are currently carrying out experiments in which IL-2-containing preparations are added to fresh PBML at the initiation of induction, in order to determine if under these conditions the PBML can achieve the rapid IFN-γ production by lymphoblasts

Also of interest is the difference in the turn-off of

IFN-γ production in these two cell types. PBML continued producing IFN for 5 days after induction, whilst IFN production in lymphoblasts was essentially complete after 24 hr. This shut-down of IFN-γ expression lymphoblasts occurred despite sustained high levels of metabolic activity. The augmentation of IFN production in lymphoblasts treated with metabolic inhibitors suggests that the shut-off mechanism requires *de novo* RNA and protein synthesis. 'Superinduction' in response to metabolic inhibitors has also been demonstrated for IL-1 and IFN-β production. In the case of IFN-β production in poly(I).poly(C)-induced fibroblasts, metabolic inhibitors are thought to prevent the production of factor(s) which degrade IFN-β mRNA (Stewart, 1979). We are currently carrying out experiments to determine whether steady-state levels of IFN-γ mRNA are maintained at higher levels for longer in lymphoblasts treated with metabolic inhibitors.

Our results clearly show that IFN-γ is expressed differently in fresh lymphocytes compared to activated lymphocytes. We hope that our continuing studies of these differences will help towards understanding the cellular and molecular modes of IFN-γ induction. It may be that differences in the state of the IFN-γ gene or genes controlling IFN-γ expression account for differences in IFN-γ production in the two cell types. There is evidence from a number of systems that the control of gene expression in mammalian cells can depend, for example, on the degree of DNA methylation (Felsenfeld & McGhee, 1982). Preliminary experiments showed that the DNA methylation inhibitor, 5-azacytidine, augmented IFN production in lymphoblasts but actually inhibited IFN production by PBML. This suggests the possiblility that DNA methylation plays a role in regulating IFN-γ gene expression.

The more rapid production of IFN-γ by restimulated lymphoblasts is at least superficially reminiscent of accelerated secondary immune responses to antigens, where memory lymphocytes respond more rapidly to antigen stimulation than do unprimed lymphocytes (Hayry & Andersson, 1974).

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