

HUMAN  $\gamma$  INTERFERON STRONGLY UPREGULATES  
ITS OWN GENE EXPRESSION IN PERIPHERAL  
BLOOD LYMPHOCYTES

BY KENNETH J. HARDY AND TAKAKO SAWADA

*From the Howard Hughes Medical Institute, Department of Medicine, Section of Clinical  
Immunology, Baylor College of Medicine, Houston, Texas 77030*

IFN- $\gamma$  occupies a central role as a potent modulator of both the effector and the effector limbs of the immune response. While promising as a therapeutic agent for immunodeficiency states, infectious and neoplastic diseases, and autoimmunity (1-3), IFN- $\gamma$  has perhaps more often been evoked in the pathogenesis of such disorders (4-9). In the latter regard particularly, recent evidence from transgenic mouse models has clearly demonstrated that overexpression of IFN- $\gamma$  can result in autoimmune destruction of tissues by inflammatory mechanisms (10). In the course of studying the cell and molecular basis for IFN- $\gamma$  gene regulation (11), we observed an IFN response consensus sequence in the most proximal 5' region of the human IFN- $\gamma$  gene very near a T cell-specific, inducible DNase I hypersensitive site 250 bp from that gene's cap site (12). Given the role of such response elements in upregulating IFN responsive genes (13, 14), we queried whether the product of the human IFN- $\gamma$  gene locus might somehow affect its own expression. In this article, definitive evidence is presented using both total human PBMCs and specific subsets of those cells, demonstrating a strong IFN- $\gamma$  autosuperinduction response to either IFN- $\gamma$  "priming" or costimulation.

Materials and Methods

For each experiment, human PBMC were isolated from 1 U of heparin-treated, random donor buffy coats (Gulf Coast Regional Blood Center, Houston, TX). All samples used were negative by HIV and hepatitis serologies. PBMC were isolated by isolymp gradient centrifugation. Mixed leukocytes were washed in HBSS and suspended in RPMI 1640, 10% FCS, penicillin/streptomycin at  $5 \times 10^6$  cells/ml before stimulation. PBMC separated into E<sup>+</sup> and E<sup>-</sup> fractions (15) were first panned in plastic petri dishes at 37°C for 45 min to remove adherent cells. For reconstitution experiments, adherent monocytes from  $3 \times 10^7$  cells were added back to equal numbers of rosetted cells before stimulation. For co-stimulation experiments, human rIFN- $\gamma$  (Genzyme Corp., Boston, MA) and PHA (final 1.0 g/ml) were added simultaneously, the mixture was incubated at 37°C for 6 h, and nonadherent cells were removed, washed three times in cold PBS, and used directly for total RNA isolation. In preincubation (primed) experiments, cells as above were first incubated with rIFN (or media alone) for 6 h at 37°C before addition of PHA. Preincubation of monocytes alone with IFN- $\gamma$  had no effect on subsequent upregulation of IFN- $\gamma$  transcripts. IFN- $\gamma$ -primed

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cells used for analysis of IFN- $\gamma$  release were washed four times in cold PBS before lectin stimulation as above. Culture supernatants were assayed for content of IFN- $\gamma$  by RIA (Centocor, Inc., Baltimore, MD). RNA was isolated by guanidine isothiocyanate disruption of cells and cesium gradient ultracentrifugation. RNA was quantified by OD<sub>2560</sub>; 15  $\mu$ g per lane was used. Ethidium staining and photographic verification of RNA load and integrity were performed in every case. Northern analyses of transcripts were determined by hybridization with cDNA probes of human IFN- $\gamma$  and  $\beta$ -actin <sup>32</sup>P labeled to 0.5–1.0  $\times$  10<sup>9</sup> cpm/g. Autoradiograms were densitometrically analyzed and computer integrated for direct comparison (Pharmacia/LKB Biotechnology, Inc., Piscataway, NJ). Cytometric analysis of total PBMC and rosette E<sup>+</sup> and E<sup>-</sup> cells used mAbs, Leu 3a, 4, 9, 11, 12, M3, and FITC-labeled goat anti-mouse (Becton Dickinson & Co., Mountain View, CA).

### Results and Discussion

To address our hypothesis that human IFN- $\gamma$  might affect its own gene expression, total human PBMC were differentially lectin activated either alone, in the presence of rIFN- $\gamma$ , or after 6 h of priming with rIFN- $\gamma$ . IFN- $\gamma$  mRNA transcripts were then measured by Northern analysis using  $\beta$ -actin as internal control. As is shown in Fig. 1, PBMC either co-stimulated or primed with IFN- $\gamma$  upregulated that gene's expression at the steady-state mRNA level. Cells primed with IFN- $\gamma$  demonstrated a significantly stronger response than those simply co-stimulated. Even subthreshold concentrations of PHA became stimulatory for IFN- $\gamma$  gene induction when as little as 10 U/ml of rIFN- $\gamma$  was present. Upregulation was even stronger, however, with higher levels of PHA (1.0 g/ml). Among different individuals, range of upregulation varied between 3- and 50-fold. IFN- $\gamma$  concentrations >500 U/ml rarely had any further effect on this upregulatory response.

Next, PBMC mock primed or primed with IFN- $\gamma$  as above were stimulated with threshold levels of PHA and assessed at various times for IFN- $\gamma$  transcripts. As is quantified densitometrically in Fig. 2, onset of IFN- $\gamma$  gene induction occurred 4-fold faster and expression was nearly 30-fold higher in IFN- $\gamma$ -primed PBMCs. High

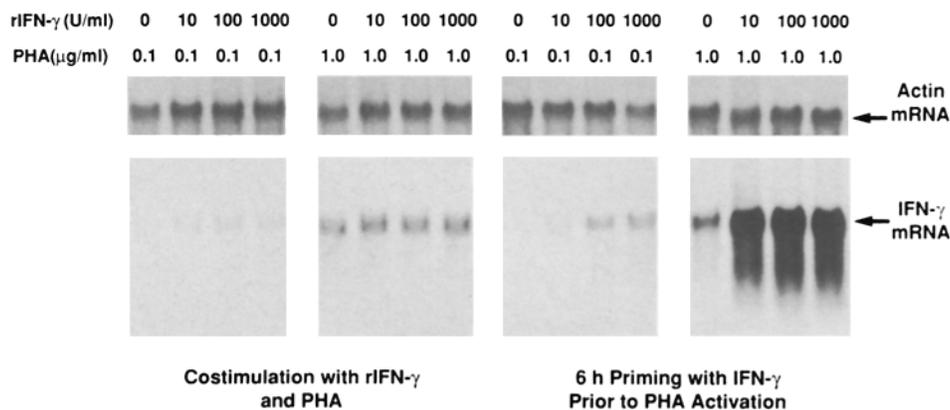


FIGURE 1. Effect of purified IFN- $\gamma$  on its own gene expression in PHA-activated PBMC. Total human PBMCs were either co-stimulated (*A* and *B*) or primed for 6 h (*C* and *D*) with rIFN- $\gamma$  at concentrations shown, then analyzed for induction of IFN- $\gamma$ -specific mRNA 6 h after stimulation with concentrations of PHA as shown. Each lane contains 15  $\mu$ g of total RNA from  $5 \times 10^7$  PBMC.

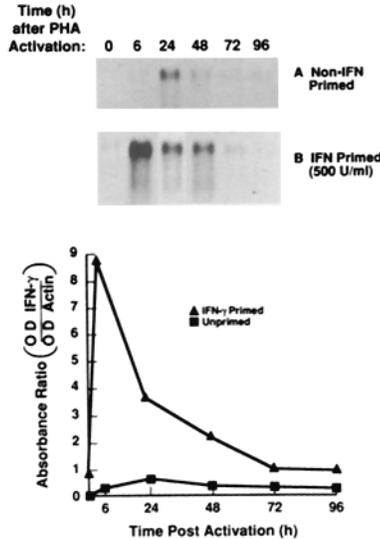


FIGURE 2. Typical kinetics of the IFN- $\gamma$  autosuperinduction response. Total PBMC were primed with rIFN- $\gamma$  (500 U/ml) for 6 h then PHA stimulated (1.0 g/ml) and disrupted at the times indicated before RNA isolation and analysis of IFN- $\gamma$ -specific mRNA. Autoradiograms in top panel represent IFN- $\gamma$  specific signal from (A) non-IFN-primed cells and (B) IFN-primed cells. Bottom panel shows densitometric quantification of these kinetics determined at each time point relative to actin-specific signal (calculated by:  $OD_{IFN-\gamma} / OD_{actin}$ ).

level expression was maintained for several days before tapering back to near control levels. Furthermore, in ~30% of PBMC samples studied to date, IFN- $\gamma$  priming resulted in some induction of IFN- $\gamma$  transcripts even in the absence of lectin stimulation (Fig. 2).

De novo synthesis of biologically active IFN- $\gamma$  after mock of IFN- $\gamma$  priming and lectin activation of PBMC was also measured using conventional RIA analysis (data not shown). Even as early as 6 h after stimulation, washed cells primed with IFN- $\gamma$  released nearly seven times more biologically active IFN- $\gamma$  into the media than did unprimed cells. This suggested that IFN- $\gamma$  priming not only upregulated IFN- $\gamma$  gene expression but also IFN- $\gamma$  protein synthesis and release.

The question of subset identification of PBMC involved in the IFN- $\gamma$  autosuperinduction response was next addressed. As is shown in Fig. 3 A, a typically strong and dose-related response was observed in total PBMCs. Surprisingly, however, rosetted T cells from the same patient whether monocyte depleted (Fig. 3 B) or repleted (Fig. 3 C) failed to demonstrate the upregulatory response, despite their normal activa-

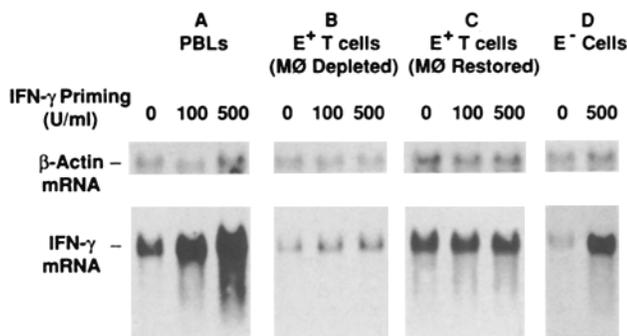


FIGURE 3. Identification of lymphocyte subset involvement in the IFN- $\gamma$  autosuperinduction response. Total PBMC (A); E<sup>+</sup>, monocyte depleted (B); E<sup>+</sup>, monocyte reconstituted (C); and E<sup>-</sup> cells (D) were primed with rIFN- $\gamma$  as indicated and IFN- $\gamma$  mRNA was assayed 6 h after stimulation with 1.0 g/ml of PHA. Control hybridization to actin is included.

tional dependence on monocytes for IFN- $\gamma$  induction. As is shown quite clearly in Fig. 3 D, however, the E<sup>-</sup> mononuclear cells are quite readily superinduced by IFN- $\gamma$  priming. Flow cytometric analysis of the E<sup>-</sup> cells revealed absence of T3<sup>+</sup> lymphocytes, ~60–70% Leu 12<sup>+</sup> (B cells), 16–25% Leu 11<sup>+</sup> NK cells, and variable (0–8%) percentages of Leu M3<sup>+</sup> (monocytes) cells depending on prior panning. These data are consistent with the notion that accessory cells (i.e., B cells and/or monocytes) in the E<sup>-</sup> fraction might themselves be the targets of IFN- $\gamma$  priming, and that these cells, or their soluble products, might indirectly be responsible for the upregulation of the NK-like cells in that fraction. However, neither supernatants from the primed E<sup>-</sup> fraction nor IFN- $\gamma$ -primed monocytes are capable of reconstituting this response when added to E<sup>+</sup> fractions. Further, total cells from the E<sup>-</sup> fraction, when added back to the E<sup>+</sup> fraction, did not reconstitute a response additively any greater than the sum of the two fractions alone (data not shown). Since only T cells and LGLs express IFN- $\gamma$  (16–18), our data suggest that the producer cells largely involved in the IFN- $\gamma$  autosuperinduction response are primarily non-rosettable mononuclear cells, presumably of the NK type. They also imply that E<sup>+</sup> lymphocytes do not participate in this response, even in the presence of various accessory cells and/or their products. Further, preliminary data from analyses of IL-2 mRNA transcripts argue against a role for IL-2 in this response.

TNF- $\alpha$  (19), IL-1 (20), platelet-derived growth factor (PDGF) (21), and both TGF- $\alpha$  and - $\beta$  (22–23) have all been recently implicated in self-amplification loops, supporting a general model involving widespread autocrine/paracrine regulation within the cytokine system. Our observations of human IFN- $\gamma$  autosuperinduction extend these earlier findings and may be particularly relevant to the pathogenesis and treatment of immunologic disease. Our findings that the response particularly involves NK-like cells may lend credence to trials of its use in combination with IL-2 in lymphokine-activated killer cell/tumor-infiltrating lymphocyte (LAK/TIL) therapy protocols. Important to autoimmune disease pathogenesis, however, is the fact that autocrine or paracrine upregulation of a potent inflammatory mediator such as IFN- $\gamma$  would be predicted to have dire inflammatory consequences unless opposed by powerful and critically regulated suppressive mechanisms. Suppression of human IFN- $\gamma$  biological activity (24) and gene expression (11) has very recently been attributed to a similarly autoinducible lymphokine, TGF- $\beta$ . It has been shown that TGF- $\beta$  is an immunosuppressive cytokine produced by rat macrophages (25). We have recently identified TGF- $\beta$  in a murine MLR supernatant and shown it to be a physiologic downregulator of murine IFN- $\gamma$  gene expression (26). Perhaps it is no coincidence that IFN- $\gamma$ , an upregulator of immune responsiveness and inflammation, is naturally opposed by TGF- $\beta$ , a downregulator of inflammation and a promoter of wound healing (27). Further insights into the cell and molecular genetic mechanisms regulating these and other members of the cytokine system should provide a foundation upon which to design more effective immunotherapeutic regimens, based on a more realistic understanding of the pathogenesis of immune dysfunction.

### Summary

The product of the human IFN- $\gamma$  gene was found to be a powerful upregulatory stimulus for its own gene expression in lectin-activated human PBMC. The IFN- $\gamma$  autosuperinduction response was further enhanced by "priming" PBMC with IFN- $\gamma$ .

Primed cells maximally upregulated their levels of IFN- $\gamma$  specific mRNA 4-fold faster and more than 20-fold higher than mock-stimulated cells. High mRNA levels persisted for several days after stimulation, and enhanced secretion of biologically active IFN- $\gamma$  paralleled the observed upregulation of gene expression. Producer cells demonstrating this response were found to be primarily localized to the rosette E<sup>-</sup> (Leu 11<sup>+</sup>) fraction of PBMC and appear to be of the LGL/NK variety. Whether the autosuperinduction phenomenon occurs through direct or indirect effects of IFN- $\gamma$  on producer cells is still unclear. These results may be important both to an understanding of the pathogenesis of immune dysfunction and to the design of more effective immunotherapy.

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