Interferon-Induced Growth Arrest Is Mediated by Membrane Structural Changes

A. GUHA,[1] R.P. MASON,[4,5] D. PRIMORAC,[2] G. KONOPASKE,[1] J. MILLER,[1] P. SINHA,[1] T.N. TULENKO,[4] and A. HAND[3]

ABSTRACT

Interferon-γ (IFN-γ) is an immunomodulator shown to augment the expression of major histocompatibility (MHC) class I/class II antigens on the cell surface. In previous studies, we have demonstrated that the enhanced expression of these antigens on the cell surface is in part due to IFN-γ-mediated abrogation of antigen shedding. In this study, we demonstrate that IFN-γ induces structural changes in the cell membrane by altering the cholesterol/phospholipid ratio. Furthermore, such changes not only mediate enhanced expression of antigen on the cell surface but may drive the cells to growth arrest and apoptosis. These results were obtained by employing x-ray diffraction, electron microscopy, and DNA analysis.

INTRODUCTION

AMONG THE THREE MAJOR CLASSES OF INTERFERONS, interferon-γ (IFN-γ) is known to be the most potent modulator of the immune system.[1–3] It is produced by Th1 cells and has been shown to enhance HLA class I and class II antigens on the cell surface.[4–7] Enhancement of these surface molecules has been shown to induce increased site density that, in turn, may accelerate target cytotoxicity.[8] Although an increase in the surface antigens by IFN-γ may be due in part to increased transcription,[7,8] our previous studies demonstrated that IFN-γ interferes with the cell surface shedding of MHC class I antigens.[4,5] These physiologic changes may mediate both positive and negative immune regulation.[9,10] Accordingly, we have demonstrated that IFN-γ abrogates hematopoiesis and erythroid differentiation.[10–13] Thus, IFN acts as a negative regulator of hematopoiesis and may obstruct hematopoietic recovery after transplants.[14] The exact mechanism by which IFN-γ modulates the immune response and controls hematopoiesis is not known. In this study, we demonstrate that reduced antigen shedding[4,5] may induce membrane structural changes in the cells that may interfere with cell proliferation and promote enhanced antigen expression. Also, abrogation of antigen shedding may modulate the composition of membranes, altering the rate of plasma membrane vesiculation and intercellular as well as intracellular signaling.

The plasma membrane consists of a lipid bilayer that not only separates the intracellular organelles from extracellular components but also maintains a fluid state and regulates dynamic physiologic functions, such as acid-base harmony, opening and closing of ion channels, and various receptor movements, as well as exfoliation of plasma membrane, particularly vesicles, that may play an important role in cell signaling.[15] Changes in the membrane components may alter the cell physiology and affect cell function.[16,17] It has been demonstrated that cholesterol is a crucial component of the membrane bilayer that controls molecular interactions and membrane fluidity, which may provide room for free movement of membrane proteins.[18,19] Thus, alteration in the cholesterol/phospholipid ratio may restrict the movement of some membrane proteins and cell surface antigens by virtue of changes in membrane fluidity.[19] In this study, we demonstrate that IFN-γ is one of the agents that may disrupt membrane fluidity and retard or abrogate normal functions of the cells, gradually driving the cell to apoptosis.

MATERIALS AND METHODS

Leukemia cell culture

The leukemia cell line A_1 (a kind gift of Dr. M.H. Freedman, Hospital for Sick Children, Toronto, Canada) was derived

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Departments of [1]Medicine, [2]Pediatrics, and [3]Pediatric Dentistry, UCONN Health Center, Farmington, CT. Departments of [4]Biochemistry and Physiology, and [5]Psychiatry, Neurosciences Research Center, MCP-Hahnemann School of Medicine, Allegheny University of Health Sciences, Philadelphia, PA.

from a child with acute lymphocytic leukemia (ALL). The cells were maintained in long-term culture in alpha medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS). A_1 cells express both major histocompatibility antigens, class I and class II. A_1 cells were treated with or without IFN-γ 1000 U/ml (Amgen, Thousand Oaks, CA) for 18, 24, 36, 48, and 72 h.[5]

Cell counts

Proliferation of A_1 cells in the presence or absence of IFN-γ (1000 U/ml) was assayed by routine cell counts and the trypan blue exclusion method. These cells were then used for either DNA extraction, x-ray diffraction, or electron microscopy.

Cell preparation for electron microscopy (EM)

A_1 cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Polysciences, Warrington, PA), pH 7.4. The cells were rinsed three times in 0.1 M cacodylate and stored overnight. The cells were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.8% potassium ferricyanide for 30 min and then rinsed three times in distilled water. Cells were pelleted and pre-embedded in agarose (Sigma Chemical Corp., St. Louis, MO), then block stained with 0.5% aqueous uranyl acetate for 1 h. After dehydration in ethanol and substitution with propylene oxide, the cell pellet was embedded in Polybed resin (Polysciences). Thin sections of the embedded cells were cut with a diamond knife, collected on copper specimen grids, stained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron microscope.

Morphometry

The sizes of cell and nuclear profiles in thin sections of treated and untreated cultured cells were determined using a digitizing tablet interfaced with a microcomputer and Sigma Scan software (Jandel, Corte Madera, CA). The data were evaluated statistically using analysis of variance (ANOVA) and t-tests for selected comparisons.

Crude plasma membrane preparation

A_1 cells, both IFN-γ treated and untreated, were collected separately from culture media, washed in PBS, and lysed by sonication.[10,20] Fractionated cell components were differentially centrifuged to deplete cytosolic and nuclear protein at 1200 and 8000 rpm, respectively. Final separation of plasma membrane fractions was obtained at 100,000g. These membrane fractions were used for the estimation of phospholipid/cholesterol ratio and x-ray diffraction studies.[18,19]

Cholesterol and phospholipid mass

The cholesterol and phospholipid mass was quantified in the plasma membrane of untreated and IFN-γ treated cells by gas liquid chromatography[18] using cholesterol methyl ether as an internal standard. Phospholipid phosphorus assay was performed using phosphatase after a chloroform/methanol extraction of lipids. Cholesterol/phospholipid mole ratio (CL/PL) was calculated as discussed by Gleason et al.[19]

X-ray diffraction

IFN-γ-treated or untreated plasma membrane fractions were centrifuged (35,000g) for 1 h at 5°C in Lucite sedimentation cells containing aluminum foil substrate. Supernatants were removed, and samples were mounted on curved glass supports. Samples were equilibrated overnight in glass vials containing saturated salt solutions that served to define specific relative humidity of 95% ($ZnSO_4$). Oriented membrane samples were then placed in sealed brass canisters containing aluminum foil windows in which temperature and relative humidity were controlled. Small angle x-ray scattering was carried out by aligning samples at grazing incidence with respect to the x-ray source, a Rigaku RU 200 rotating copper anode microfocus generator (Danvers, MA). The fixed geometry used a single Franks mirror providing nickel-filtered radiation. The x-ray diffraction patterns were recorded on a one-dimensional position-sensitive electronic detector (Innovative Technologies Inc., Newburyport, MA).[21] The error associated with this measurement is less than 0.4 Å, corresponding to instrumental uncertainty. Additional details related to the statistical evaluation of the small angle x-ray diffraction data have been discussed previously.[21]

Genomic DNA extraction

Genomic DNA extraction was performed as described,[20] with some modification. Briefly, cells (6×10^6–1×10^9) treated or untreated with IFN-γ were placed in Falcon 10 ml tubes containing 5.0 ml 2 × SET (10 × extraction buffer: 10.0% SDS, 0.5M Tris HCl, pH 7.5, 10 mM EDTA) and 200 μg/ml proteinase K and rocked overnight at 55°C. The mixture was extracted in an equal volume of phenol/chloroform/isoamyl alcohol (12:12:0.5 by volume). The aqueous portion was adjusted with 0.3 M ammonium acetate, incubated at ambient temperature for 10 min, and centrifuged at 10,000g for 10 min. Two to three volumes of ethanol were layered over the extract, and DNA was centrifuged at 13,000g for 10 min, washed twice with 70% ethanol, air dried, and redissolved in 1.0 ml Tris-EDTA buffer, pH 8.0.

Agar gel electrophoresis

Ultrapure agarose (GIBCO BRL, Grand Island, NY) (1.8 g) was dissolved in 1 × TBE (Bio-Whittaker, Walkersville, MD) and poured into a 4 × 4 gel trough (Biorad, Hercules, CA). Electrophoresis was conducted for 2 h at 100 V.

RESULTS

Effect of IFN-γ on cell proliferation

A_1 cells were washed, counted (5×10^5–1×10^6/ml), resuspended in alpha medium with or without IFN-γ (1000 U/ml), and cultured in the CO_2 incubator for 24, 48, and 72 h. At the end of each interval, the cells were counted, and live and dead cell ratios were determined by trypan blue exclusion. Untreated cells proliferated, and cell number was approximately doubled in 48 h. IFN-treated cells proliferated slowly and demonstrated significantly more cell death as compared with time-matched untreated cells. These results suggest that IFN-γ not only may abrogate cell proliferation but also induces cell death over time. The p value was determined by Student's t-test (Table 1).

TABLE 1. EFFECTS OF IFN-γ ON CELL DIVISION/DEATH[a]

Sample	Time (h)	No. of cells ($\times 10^5$) Live	Dead	% dead cells
A$_1$	0	5	—	—
Untreated	24	6[b]	0.1	1.6
IFN-γ	24	5[b]	0.2	3.8
Untreated	48	9[c]	0.2	2.2
IFN-γ	48	7[c]	0.2	2.8
Untreated	72	22[d]	2.7	10.9
IFN-γ	72	13[d]	2.1	13.9

[a]These experiments were repeated more than 5 times and a mean of the experimental data is projected in this table. Values with the same letters are significantly different.
[b]$p \leq 0.001$; [c]$p \leq 0.001$; [d]$p \leq 0.001$ (t-test).

Electron density profile of a plasma membrane fraction after treatment with or without IFN-γ

A$_1$ cells were treated with or without IFN-γ and cultured for 24 or 48 h, at which time cell counts were performed to estimate cell proliferation and cell death. Both treated and untreated cells were washed in PBS and were lysed by sonication.[9,10] Plasma membranes were separated by differential centrifugation as described[9–12] and prepared for x-ray diffraction studies. Representative membrane electron density profiles are shown in Figure 1. The membrane width, or d-space, is the measured distance from the center of one membrane to the next, including surface hydration. The measurement corresponds to the stacked or membrane multilayer geometry used for the diffraction experiments. The d-space measurement represents an average width for each membrane in the multilayer structure. Plasma membrane d-space measurements were 66.7 Å for untreated cells and 67.5 Å for IFN-γ treated cells, suggesting that the profile of IFN-treated plasma membrane was significantly altered. After IFN treatment, a marked increase (3–4 Å) in intrabilayer phosphate head group separation was observed in the samples. A broad increase in electron density in the membrane-hydrocarbon core was also observed after IFN-γ treatment (Fig. 1).

Cholesterol/phospholipid ratio in A$_1$ cells cultured with or without IFN-γ

A$_1$ cells were cultured in alpha medium in the presence or absence of IFN-γ for 4 h or 24 h and used for determining the cholesterol/phospholipid ratio. The results demonstrate that even 4 h (data not shown) treatment with IFN-γ increases the membrane cholesterol/phospholipid ratio, and this increase continues over time (Table 2). These experiments were repeated several times to confirm our observations. Significance was tested by comparing the mean percent change in the CL/PL for each experiment to determine if the mean percent was significantly different from zero using Student's t-test.

Morphologic changes induced by IFN

The leukemia cell line A$_1$ was cultured in the presence or absence of IFN-γ for 24 and 48 h and prepared for electron mi-
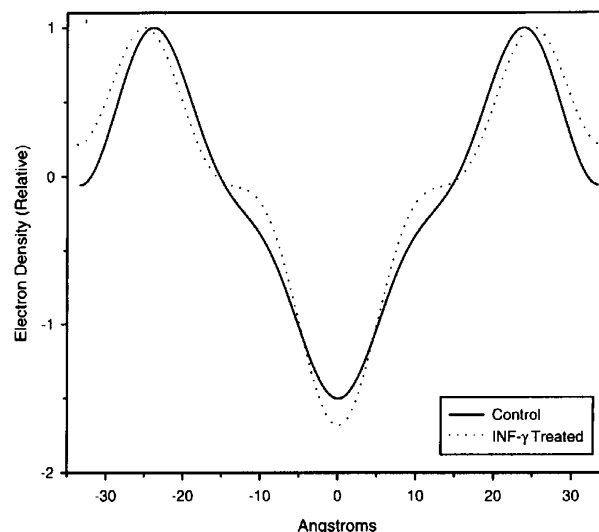

FIG. 1. One-dimensional electron density profiles (Å) of an enriched plasma membrane fraction of control cells (solid line) and after IFN treatment (dashed line). The peaks of electron density correspond to the phospholipid head groups, as the minimum of electron density at the center of the bilayer correlates with the terminal methylene segments. The d-space measurements for these samples were 66.7 Å (control) and 67.5 Å (IFN treated). The experiments were carried out at 96% relative humidity and 25°C.

croscopy. Untreated control cells were roughly spherical in shape, with few plasma membrane ruffles or projections (Fig. 2A). The nuclei were oval to spherical in shape, occasionally exhibited shallow indentations, and contained finely granular chromatin and, typically, one or two nucleoli. The cytoplasm contained numerous mitochondria, a few cisternae of rough endoplasmic reticulum, a moderately developed Golgi apparatus, and occasional lipid droplets. After culture in the presence of IFN, the majority of cells appeared similar to untreated cells. An increased number of cells, however, showed changes typical of apoptosis (Fig. 2B,C). These changes included condensation and margination of nuclear chromatin, nuclear fragmentation, cytoplasmic vacuolation and loss of organization, and increased plasma membrane ruffling.

Changes in cell and nuclear size as a result of IFN treatment were evaluated by measurement of cell and nuclear profile ar-

TABLE 2. CHOLESTEROL/PHOSPHOLIPID RATIOS IN IFN-γ-TREATED CELLS

Cells	n	Cholesterol/phospholipid Mean	SD	SEM
Control (untreated)	5	0.386	0.103	0.046
24 h IFN-treated	5	0.442[a]	0.106	0.047

[a]$p < 0.03$ compared with control (t-test).
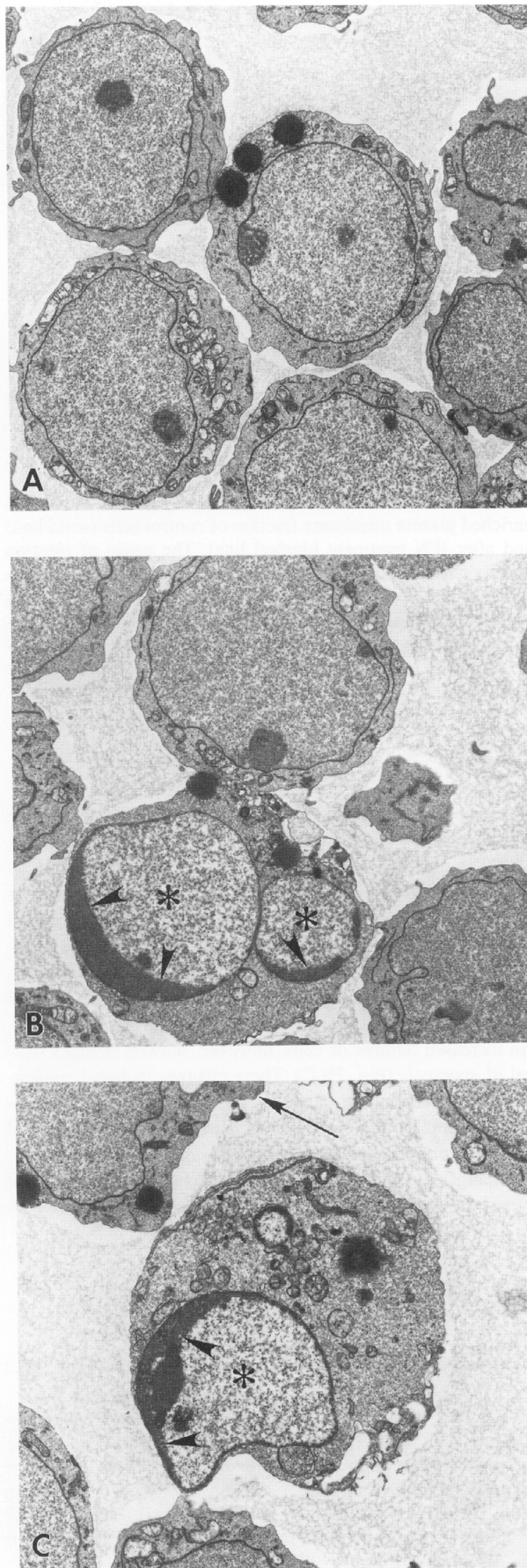
eas in random sections of cell pellets (Table 3). After 24 h of culture, both treated and untreated cell profiles were significantly smaller than cells fixed just before culture. Nuclear profiles of untreated 24 h cultured cells were not significantly smaller than uncultured cells, whereas the nuclei of IFN-treated cells were significantly smaller than those of untreated cultured cells.

Analysis of DNA extracted from A_1 cells

A_1 cells were cultured in the presence or absence of IFN-γ for 18, 24, 36, and 48 h. DNA was isolated from the cells as described in Materials and Methods. Extracted DNA was resolved on 0.8% agarose gels (Fig. 3). Our results indicate that DNA extracted from IFN-γ-treated cells was fragmented at 900 and 1500 bp, as opposed to untreated control cells. Although these fragmentations were also visible for DNA extracted from time-matched, untreated cells (data not shown), the specific sites were more intense in IFN-γ-treated cells with time. These results suggest that IFN-γ may enhance the site-specific DNA fragmentation of A_1 cells.

DISCUSSION

IFN-γ has been shown to be both a positive and negative regulator of immune mechanisms.[4,11] We have demonstrated that IFN-γ enhances cell surface antigens, such as HLA class I, and cytokines, such as mBPA,[9] on the cell surface[4,5,10] by retarding antigen turnover,[4] with the result that exfoliation of surface antigens may be slowed or abrogated.[17] Although we have observed that synthesis of these antigens continues at a normal rate for the first few hours,[4] cells treated for even a short time (as short as 4 h) with IFN-γ have an increased antigen-specific site density, probably because of retarded antigen shedding.[4] The enhanced expression of such antigens as HLA may render the cells more susceptible to lysis by CTL.[5] Subsequently (8–16 h later), there is an increase in mRNA for MHC antigens as the synthesis continues at a normal rate, whereas the antigen turnover is retarded. It is possible that observed increase in mRNA is caused by the continual synthesis of the specific proteins, as described above MHC in this case.[4,5] However, we contend that these changes may exert extra stress on the cell surface and thus may alter the composition of the cell membrane (Fig. 1), which may further bring about other physiologic changes, including growth arrest, apoptosis, and cell death (Fig. 2 and Table 1).

In this study, we demonstrate by x-ray diffraction analysis that there is a change in the membrane bilayer width of the cells treated with IFN and a marked increase in intrabilayer phos-

FIG. 2. Electron micrographs of A_1 cells cultured for 24 h in the absence or presence of IFN. Untreated cells (A) are spherical, with nuclei containing finely granular, dispersed chromatin. In IFN-treated cultures (B,C), increased numbers of cells are undergoing apoptosis, with condensed, marginated chromatin (arrowheads), fragmented nuclei (*), cytoplasmic vacuolation and loss or organization, and plasma membrane ruffling (arrow). (A) ×5500; (B) ×6300; (C) ×18,000.

TABLE 3. EFFECTS OF IFN-γ ON CELL AND NUCLEAR PROFILE SIZE

	Nuclei				Cells			
Sample	n	Mean (μm²)	SD	SEM	n	Mean (μm²)	SD	SEM
0 h	30	58.23[a]	37.91	6.92	28	102.7[a,b]	74.89	14.15
24 h untreated	38	44.26	20.97	3.40	42	75.71[a]	28.32	4.37
24 h IFN treated	38	34.75[a]	21.72	3.52	32	69.10[b]	25.78	4.56

[a,b]Values with the same letter are significantly different ($p < 0.05$) (t-test).

phate head group separation (Fig. 1). The change in membrane structure observed by x-ray diffraction is consistent with a statistically significant increase in the cholesterol/phospholipid ratio (Table 2). We propose that an increase in cholesterol levels after IFN-γ treatment may increase the rigidity of membrane structure and change membrane fluidity, with the result that the surface antigens may be welled in the membrane bilayer and, in turn, may restrict free movement of membrane proteins and contribute to retarded antigen turnover.

It is important to understand that any physiologic change that alters the membrane structure and function may also alter cell physiology and cell morphology. Our electron microscopy results support this concept and suggest that IFN-γ treated cells exhibit morphologic changes similar to those characteristic of apoptosis. Chromatin condensation of the nucleus (Fig. 2) and ruffling of the membrane are observed occasionally in the population treated with IFN-γ, although when nuclear fragmentation is observed by agarose gel electrophoresis, the patterns fail to represent classic nuclear fragmentation. The intensity of naturally occurring bands in cultured cells is shown to be enhanced by IFN treatment, suggesting that IFN-γ may enhance the rate of apoptosis by targeting fractionation of DNA at specific sites,

which may be demonstrative of a preapoptotic stage. These results suggest that IFN-γ may act on certain regions of genomic DNA, resulting in the band sizings (mol wt ~1.5 bp, ~900 bp) rather than inducing the classic fragmentation patterns observed in apoptosis. It is also possible that these fragmentation patterns may be dictated by cell cycle/cell division or may be different in different cell types. Furthermore, this finding supports the premise that IFN-γ may act on the physiology of cell division and DNA replication rather than directly on DNA fragmentation. As our proliferation assay (data not shown) and cell count assay (Table 1) indicate that IFN-γ abrogates cell proliferation and enhances cell death, it is possible that changes observed in DNA patterns are mediated by growth arrest rather than DNA fragmentation, as demonstrated by the size of the cell and nuclear profiles (Table 3) or may simply represent a preapoptotic stage. Further studies in this area are in progress in our laboratory.

It is interesting to note that although enhanced expression of antigens on the cell surface, as shown in our previous work,[4,5] may make the cell immunologically more competent for target lysis, the same cells are unable to turn over their antigens and retard shedding.[5] Alteration of important physiologic
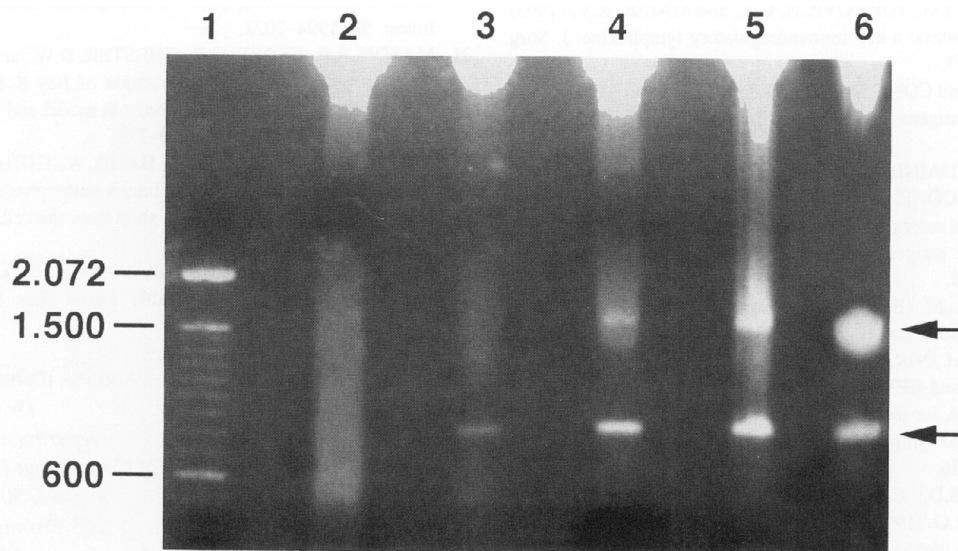

FIG. 3. Analysis of genomic DNA after treatment with IFN-γ. Lane 1, 100 bp DNA ladder. DNA extracted from cells before treatment (lane 2). DNA extracted from the cells treated with IFN-γ (lanes 3, 4, 5, 6 for 18, 24, 36, and 48 h, respectively) demonstrates two fragments of 1500 and 900 bp. Equal concentration of DNA was loaded in each lane. However, the intensity of the observed DNA fragments increases with the length of incubation with IFN-γ.

processes, such as shedding and cell proliferation (Table 1), may induce cellular distress, and this very process may act as a negative regulator. Shed antigens from the cell surface, especially such cytokines as mBPA, GM-CSF, and others,[10,22,23] which have been shown to stimulate hematopoiesis, may be negatively affected when shedding is disrupted. Consequently, abrogation of such physiologic events may have a negative effect on hematopoiesis not only by means of retarded cell proliferation of hematopoietic cells but also by means of reducing the available shed antigens (both soluble and vesicle bound). These events have been shown to have an important physiologic role in the regulation of hematopoiesis.[10,21] From these results, it is possible to conjecture that IFN-γ is an important immunoregulator, both positive and negative, whose role in immune manipulation and hematopoiesis is not fully understood.

ACKNOWLEDGMENTS

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Address reprint requests to:
Dr. Amala Guha
Department of Medicine
University of Connecticut Health Center
Room L-5053, MC 1315
263 Farmington Avenue
Farmington, CT 06030-1315