ANALYSIS OF HUMAN BLOOD MONOCYTE ACTIVATION AT THE LEVEL OF GENE EXPRESSION

Expression of Alpha Interferon Genes During Activation of Human Monocytes by Poly IC/LC and Muramyl Dipeptide

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Human monocytes have been shown to be critical regulatory cells in the immune response. Their functions include antigen presentation (1), the generation of cytolytic T lymphocyte responses (2, 3), and various accessory cell functions (4). Monocytes have a number of other effector functions, including the performance of antibody-dependent cellular cytotoxicity (5), and spontaneous tumoricidal activity (6, 7). Monocytes exert a number of their stimulatory and modulatory activities on other components of the immune response by releasing highly bioactive molecules, including prostaglandins (8), interleukin 1 (IL-1) (4, 9), α-interferon (IFN-α)[1] (10, 11), and superoxide (O_2^-) (12). In addition, monocytes are known to also be capable of secreting dozens of other potent cytokines (13), including fibroblast growth factor(s) (FGF) (14, 15).

Our current understanding of monocyte and macrophage activation centers more around the nature of the effective stimuli for functional changes than around the regulatory mechanisms responsible for the development of these functions (16, 17–19). In view of the potential clinical importance of understanding the mechanisms regulating the expression of different aspects of the monocyte functional repertoire, we have begun an investigation of the molecular basis of monocyte activation. Here we report the results of studies on IFN-α production and release in human monocytes after exposure to two different activation agents, poly IC/LC and muramyl dipeptide.

Materials and Methods

Leukapheresis Technique. Leukapheresis was performed on normal volunteers for 2 h on a Celltrifuge II apparatus (Fenwal Laboratories, Deerfield, IL) after informed consent

[1] *Abbreviations used in this paper:* FGF, fibroblast growth factor; HuIFN, human interferon; IFN, interferon; MDP, muramyl dipeptide; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate; TCA, trichloroacetic acid.

as previously described (20). We eliminated donors who had any history of atopy or other immunologic disorders, drug ingestion of any sort (including aspirin or birth control pills), or abnormalities on physical or laboratory examinations.

Monocyte Isolation. Monocytes were isolated from the above leukapheresis specimens by countercurrent centrifugal elutriation as previously described (21). Briefly, leukapheresis specimens were passed over standard Ficoll-Hypaque gradients to produce an unfractionated mononuclear leukocyte suspension. These leukocyte cell preparations were then suspended in elutriation medium (RPMI 1640 [Flow Laboratories, Inc., McLean, VA] with 2 g/100 ml of human clinical grade human albumin [Cutter Laboratories, Inc., Berkeley, CA] with a final pH of 7.4) and entered with a Sarns cardiovascular pump into a Beckman JE6 elutriation chamber and rotor system (Beckman Instruments, Inc., Palo Alto, CA) at an initial medium flow rate of 5 ± 0.4 ml/min. The elutriation rotor speed was maintained at $2,020 \pm 10$ rpm, and the centrifuge temperature was held at 18°C. Elutriation medium leaving the elutriator rotor was collected in 50-ml aliquots in polypropylene conical centrifuge tubes (Corning Glass Works, Corning, NY), and the cells in each aliquot were sized by a Coulter H4 channelizer system (Coulter Electronics, Hialeah, FL). The flow rate of medium was gradually increased until equal numbers of lymphocytes and monocytes were seen to be leaving the separation chamber (as determined by Coulter channelizer analysis), and the 50-ml aliquot with the above-described 50:50 lymphocyte/monocyte ratio became the penultimate aliquot of the isolation procedure. Purified monocytes left in the rotor were then collected by stopping the rotor while maintaining constant medium flow rate. All of the fractions containing lymphocytes alone were pooled to obtain purified lymphocytes. The final medium flow rate, 12 ± 0.4 ml/min, and the total number of 50-ml aliquots collected (from 9 to 15) varied depending on the elutriation characteristics of each donor's mononuclear leukocytes. An average of 5 h elapsed from the start of leukapheresis until the final isolation of purified monocytes. All medium used was endotoxin free (<0.1 ng/ml of endotoxin, by limulus assay) and no antibiotics were added to any stage of the monocyte isolation procedure.

Monocyte and Lymphocyte Identification Procedures. The purity of the monocyte and lymphocyte preparations obtained by elutriation was confirmed by morphology on Wright's-stained cytocentrifuge preparations, by nonspecific esterase staining (22), and by the ability to ingest latex particles. Viability was determined to be >98% by trypan blue dye exclusion. The average purity of the monocytes used in this study, by Wright's staining, was 93%; by esterase staining, 92%; and by latex ingestion, 93%. The average number of monocytes obtained per donor was 650 million (data not shown).

Serum-free Medium. Human monocytes were cultured and activated in a chemically defined, serum-free medium as previously described (23). This consisted of Iscove's modified Dulbecco's medium supplemented with human serum albumin (fatty acid free, 4 mg/ml), cholesterol (>90% pure, 20 mg/ml), L-α-phosphatidylcholine (80 U/ml), and human transferrin (98% pure, 1 U/ml), all from Sigma Chemical Company (St. Louis, MO); insulin (0.128 U/ml; Eli Lilly and Co., Indianapolis, IN); ferrous chloride (7 × 10^{-11}; Fisher Scientific Co., Pittsburgh, PA); and β-mercaptoethanol (10^{-7} M; Eastman Kodak, Rochester, NY). The L-α-phosphatidylcholine and cholesterol were prepared together and sonicated with a Branson sonifier (Branson Sonic Power Co., Danbury, CT) with a microtip at a setting of 6, for 60 min at 4°C. This preparation, like the others, was then filtered (0.45 μm; Nalge Co., Rochester, NY) and stored at −20°C.

Monocyte Culture and Activation. Human, elutriator-purified monocytes were cultured in custom-designed, flat-bottomed Teflon labware (Corning Glass Works) capable of holding 2–40-ml volumes of fluid. Monocytes were suspended at 1 million cells/ml in the above-described, serum-free medium, placed in the Teflon plates, and then rocked (7 cycles/min) in a 37°C, 5% CO_2 incubator. For activation of IFN-α secretion, monocytes were stimulated for 4 h with 10–200 μg/ml of polyriboinosinic acid/polyribocytidylic acid (2 mg/ml), poly-L-lysine (1.5 mg/ml), and sodium carboxymethylcellulose (5 mg/ml) (pH, 7.8) (poly IC/LC) (lot UI82-105; University of Iowa College of Pharmacy, Iowa City, IA). To activate FGF release, monocytes were stimulated for 4 h with 15–150 μg/ml of muramyl dipeptide (MDP) (Calbiochem-Behring Corp., La Jolla, CA). At the end of 18 h

of culture and after determining cell counts and viability with trypan blue as previously described (22), we harvested both stimulated and unstimulated monocytes for RNA separation. For the monokine-release assays (IFN-α and FGF), monocytes were cultured as above for 18–72 h, the supernatants were harvested, and monokine assays were performed on supernatants.

IFN-α Bioassay. IFN-α activity of culture supernatants was determined by Biofluids, Inc. (Rockville, MD) in microtiter plates by inhibition of the cytopathic effect on human foreskin cells infected with vesicular stomatitis virus and is expressed in reference standard units (10). Reference human IFN-α (HuIFN-α) was supplied by the National Institute of Allergy and Infectious Diseases (NIAID). IFN-α activity was confirmed by assaying for pH 2 stability and by neutralization with a sheep anti–human leukocyte IFN antibody (NIAID Research Resources Branch Cat. No. G026-502-568 [10]); this antiserum has been shown to inhibit 95% of the activity of a recombinant HuIFN-α preparation, but <10% of the activity of a recombinant HuIFN-β preparation.

IFN-α Bioassay on Cell Lysates. To determine whether bioactive IFN-α was being synthesized in the cell cytoplasm, 2.5×10^7 control or activated monocytes were cultured as described above. At the end of 36 or 72 h, the cells were extensively washed and suspended in 1 ml of RPMI 1640. The cell suspension was then lysed by freeze-thawing (twice), and cellular debris was pelleted by centrifugation at 1,000 g for 15 min. The supernatant was then assayed for IFN-α activity as described above; the lower limit of sensitivity of this assay is 120 U of IFN-α activity per milliliter of cell cytoplasm. Data are expressed in units defined as the IFN-α activity calculated to be in 1 ml of cytoplasm obtained from 10^9 cells.

FGF Assay. Supernatants were assayed for FGF activity by a fibroblast proliferation assay as recently described (24). Fibroblast proliferation was measured as the amount of trichloroacetic acid (TCA)-precipitable [^{3}H]thymidine (6.7 μCi/mmol) (New England Nuclear, Boston, MA) counts incorporated into fibroblasts after a 24-h exposure of each well (10^4 cells) to 1 μCi of [^{3}H]thymidine.

RNA Extraction Procedure. For each data point tested, 400 million purified human monocytes were harvested after 18 h of culture. Total cellular RNA was extracted from these cells by the guanidinium isothiocyanate/cesium chloride extraction method of Ullrich et al. (25). Poly(A)enriched RNA was obtained by passing total RNA over oligo(dT) columns as previously described (26).

Gel Electrophoresis and Northern Transfer of Messenger RNA. Messenger RNA extracted from 400 million human monocytes was separated by gel electrophoresis (~2 μg/sample well) using glyoxal as the denaturing agent and a 1.0% agarose gel as described by Thomas (27). After 8 h of electrophoresis, the gel was blotted onto nitrocellulose sheets by the Northern transfer technique as previously described (27). Ribosomal RNA size markers from *Escherichia coli* (16S, 1.5 kb; 23S, 2.9 kb) and human monocytes (18S, 1.7 kb; 28S, 4.8 kb) were electrophoresed in a parallel lane. Alternatively, the poly(A)enriched RNA from 100 million monocytes or lymphocytes was dot-blotted directly onto nitrocellulose sheets as previously described (27); denatured IFN-α cDNA probe was used as the positive control. Serial fourfold dilutions of the plasmid DNA and RNA samples were used.

IFN-α cDNA Probes. pLM001, a subclone of a previously described (28) cDNA probe for human IFN-α_1 (pAS-1), which was developed by Dr. Lester May (currently at Stony Brook University in New York) while working at Enzo Biochemicals (New York) in association with The Rockefeller University, New York, was kindly provided to us by Dr. Pravinkumar Sehgal (The Rockefeller University). In addition, through the kindness of Dr. Richard Derynck (Genentech, Inc., South San Francisco, CA), we used a second previously described IFN-α_2 cDNA probe (HuIFN-α_2) (29). These IFN-α cDNA probes were labeled with [^{32}P]dCTP and [^{32}P]dATP (>400 Ci/mmol; Amersham Corp., Arlington Heights, IL) by nick translation, as previously described (30), at a specific activity of 2–3 $\times 10^8$ cpm/μg DNA.

RNA Blotting and Hybridization Procedure. Monocyte RNA was transferred from the 1.0% agarose gel to nitrocellulose sheets as previously described (27). Hybridization of the nick-translated cDNA probes to the RNA on nitrocellulose sheets was performed

under the following relaxed conditions: the 24-h prehybridization and 72-h hybridizations were performed in 6× standard sodium citrate (SSC) (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's (1× Denhart's is 0.02% polyvinyl pyrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin [fraction 5]), 50% formamide, 100 μg/ml of yeast RNA, and 40 μg/ml of sheared, single-stranded salmon sperm DNA, at 37°C. Blots were hybridized with 1 × 10⁷ cpm of labeled probe (boiled for 10 min). Blots were then washed four times in 2× SSC, with agitation, for 5 min each at room temperature, followed by washing twice in 0.5× SSC plus 0.1% sodium dodecyl sulfate (SDS) for 20 min at 45°C. Blots were exposed on either Kodak XAR-5 or DuPont Cronex-4 x-ray film, with DuPont Lightning-Plus intensifying screens, at −70°C for 72 h.

Statistics. The Student's *t* test was used to analyze the significance of certain of our data.

Results

Production of IFN-α and FGF by Activated Human Monocytes. Fig. 1 demonstrates the amount of IFN-α released by monocytes at 36 h when cultured in serum-free medium under three sets of conditions: unstimulated, after exposure to 10–200 μg/ml of MDP, or after exposure to 10–200 μg/ml of poly IC/LC. Although unactivated monocytes and MDP-treated monocytes did not secrete IFN-α, poly IC/LC induced a dose-responsive secretion of IFN-α, up to 5.5 × 10² U/ml. IFN-α secretion occurred as early as 18 h and as late as 72 h (data not shown); the maximal rate of secretion was observed at 36 h. In contrast, whereas both control and poly IC/LC–stimulated monocytes produced low baseline levels of FGF, MDP-stimulated monocytes (200 μg/ml) produced approximately four times the baseline amount of FGF at 36 h after stimulation (Fig. 2).

RNA Extraction. Monocytes were harvested after 18 h of culture and the total RNA was harvested by the guanidinium/cesium chloride isolation technique. This time point was selected because it corresponded to the initiation of IFN-α release. There was no significant difference in the overall recovery of RNA
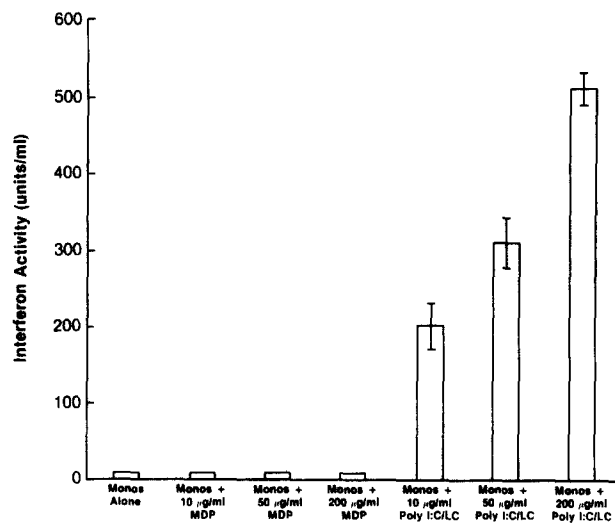

FIGURE 1. Human monocytes were cultured alone or with 10–100 μg/ml of MDP or 10–200 μg/ml of poly IC/LC. Supernatants were harvested at 36 h and IFN-α activity measured. Data represent the mean and standard error of three separate experiments.
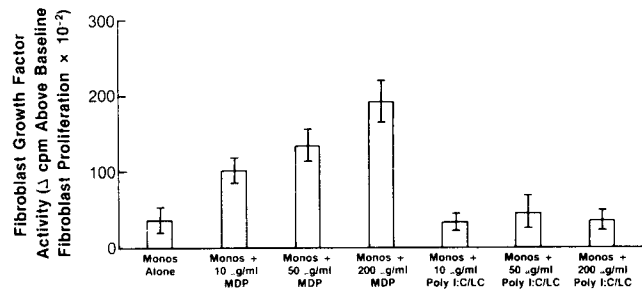
FIGURE 2. Human monocytes were cultured alone, with 10–200 µg/ml of poly IC/LC or 10–200 µg/ml of MDP. Supernatants were harvested at 36 h and incubated with 10⁴ cultured fibroblasts per well to determine FGF activity by [³H]thymidine incorporation. Data represent the mean and standard error of three separate experiments. Mean baseline proliferation for fibroblasts without added factors was 1.25 × 10⁴ TCA-precipitable cpm/10⁴ cells.
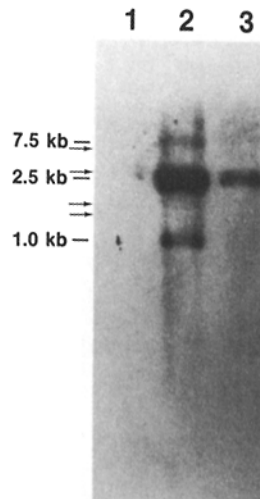

FIGURE 3. A representative autoradiograph of messenger RNA from monocytes after gel electrophoresis, Northern transfer onto nitrocellulose, and hybridization for 72 h with a ³²P-labeled cDNA for IFN-α (pLM001). (1) Unstimulated monocytes; (2) monocytes cultured for 18 h after stimulation with 200 µg/ml of poly IC/LC; (3) monocytes stimulated with 200 µg/ml of MDP. The messenger RNA (~2 µg) from 400 million purified cells was loaded into each lane. Band size calculations (1.0–5.5 kb) are numerically indicated on the left; the actual migration of size markers (1.5 and 2.9 kb E. coli and 1.7 and 4.9 kb human monocyte ribosomal RNAs) are indicated by arrows in the left margin.

between the unactivated vs. MDP-activated or poly IC/LC–activated human monocytes (~110 µg of total RNA per 10⁸ monocytes [data not shown]).

Hybridization of IFN-α cDNA Probes to Human Monocyte Messenger RNA.   Fig. 3 demonstrates a representative autoradiograph of glyoxal-denatured human monocyte poly(A)enriched RNA that has been separated by agarose gel electrophoresis, blotted to nitrocellulose sheets, and hybridized to a ³²P-labeled IFN-α cDNA probe (pLM001). The results indicated that, after 18 h of culture, control (unactivated) monocytes (Fig. 3, lane *1*) did not produce detectable levels of IFN-α mRNAs that were hybridizable to the IFN-α cDNA probe used. In
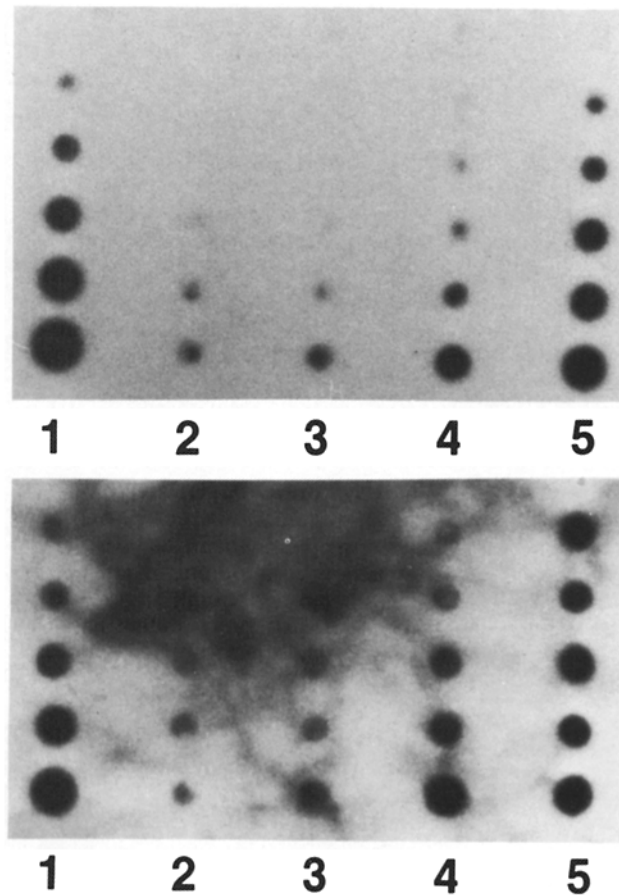
FIGURE 4.   A representative autoradiograph of serial fourfold dilutions of messenger RNA from monocytes hybridized for 72 h with either of two ^{32}P-labeled cDNA for IFN-α after dot blotting onto nitrocellulose. (A) Hybridization with pLM001. (B) Hybridization with HuIFN-α_2. (1) 0.1 μg of denatured cDNA probe (pLM001 for A, HuIFN-α_2 for B); (2) poly A–selected RNA from 100 million unstimulated monocytes; (3) poly A–selected RNA from 100 million monocytes stimulated with 10 μg/ml of poly IC/LC; (4) poly A–selected RNA from 100 million monocytes stimulated for 18 h with 50 μg/ml of poly IC/LC; (5) poly A–selected RNA from 100 million monocytes stimulated with 200 μg/ml of poly IC/LC. 250 ng of poly A–selected RNA was spotted in the first dot position of lanes 2–5, followed by four, serial, fourfold dilutions.

contrast, monocytes activated with both poly IC/LC and MDP contained detectable IFN-α mRNAs. Monocytes activated with poly IC/LC (Fig. 3, lane 2) expressed the 1.0 kb mRNA normally associated with secreted IFN-α (28). In addition, two higher molecular weight, novel RNA species (2.8 and 5.5 kb) were detected. Monocytes activated with MDP (Fig. 3, lane 3) contained only 2.8 kb mRNA. Similar results were observed in three separate experiments using different normal volunteers (data not shown).

Dot blot hybridization (Fig. 4) revealed a dose-responsive expression of IFN-α mRNA in poly IC/LC–stimulated monocytes; as the poly IC/LC concentration was increased from 10 to 200 μg/ml, the size of the dots also increased. Similar

TABLE I

IFN-α Activity* Found in the Cell Lysates of Cultured Monocytes at 36 h and 72 h

	Unstimulated		Poly IC/LC stimulated[‡]		MDP stimulated[§]	
	36 h	72 h	36 h	72 h	36 h	72 h
Antiviral activity	<120	<120	1050 ± 250[ǁ]	810 ± 180	350 ± 35	230 ± 75
Antiviral activity after pH 2 neutralization	NT[¶]	NT	1,020**	945	360	400
Antiviral activity after anti-IFN-α antibody neutralization	NT	NT	<120	<120	<120	<120

* Expressed as calculated units per milliliter of lysate (10^9 cells).
[‡] 200 μg/ml of poly IC/LC.
[§] 200 μg/ml of MDP.
[ǁ] Represents mean ± SEM of four separate experiments.
[¶] Not tested.
** Average of two experiments.

results were seen when the dot blots were probed with pLM001 (Fig. 4A) or the HuIFN-α2 probe (Fig. 4B).

Synthesis of Cytoplasmic IFN-α by Human Monocytes. In an attempt to evaluate whether MDP-activated monocytes (expressing 2.8 kb IFN-α mRNA) might be synthesizing an unreleased cytoplasmic form of IFN-α, extensively washed control and activated monocytes were lysed at 36 and 72 h, and IFN-α measurements performed on the cell lysate. As shown in Table I, control (unactivated) monocyte cell lysates were consistently found to be free of IFN-α activity at both time points. The mean IFN-α activity found in the cell lysates of poly IC/LC-stimulated monocytes (four experiments) was 1,050 ± 250 U/ml at 36 h. The mean IFN-α activity found in the monocyte cell lysates after 36 h of MDP stimulation (350 ± 35 U/ml) was significantly lower ($P < 0.05$). Intracellular IFN-α levels at 72 h after either poly IC/LC or MDP stimulation were slightly lower than the levels observed at 36 h. This intracellular IFN-α activity was pH 2 stable and neutralizable by anti–human leukocyte IFN antiserum. It was not possible to determine if any of the intracellular IFN-α activity found in the poly IC/LC-stimulated cells was of the exclusively intracellular variety; some of the intracellular activity was likely being processed for secretion.

Discussion

We detected two species of IFN-α mRNA with IFN-α probes in poly IC/LC-stimulated human monocytes that we did not detect in unstimulated monocytes or in MDP-stimulated monocytes. The 1.0 kb band is similar to the major size class for IFN-α described by others for lymphoblastoid cell lines (28). The detection of higher molecular weight bands is also consistent with these previous studies. The relative proportions of the low and high molecular weight IFN-α mRNA forms of the stimulated human monocytes observed here seem quite different from those observed in Sendai virus–induced Namalwa cells (28). This may indicate that the expression of the IFN-α family of genes differs in monocytes vs. lymphoblastoid cell lines and perhaps varies with the stimulatory agents used in the two sets of experiments. We assume that the 2.8 kb and 5.5 kb species

detected here represent different primary transcripts, since the IFN-α genes lack introns and therefore should not be subject to intron splicing. The origin of these high molecular weight species requires more direct investigation.

Human monocytes activated by either MDP or poly IC/LC also contained a 2.8 kb IFN-α mRNA. In human monocytes activated by MDP, which secrete FGF but not IFN-α, the 2.8 kb mRNA was the only IFN-α mRNA detected under the hybridization conditions used in these experiments. The presence of a 2.8 kb IFN-α mRNA appears to be associated with the presence of an intracellular antiviral reactivity that was neutralizable by an antiserum specific for IFN-α, and was pH 2.0 stable. However, we cannot exclude the possibility that very low amounts of another IFN-α mRNA (such as the 1.0 kb mRNA for IFN-α) may have gone undetected in the MDP-stimulated monocytes. It should be noted further that the 2.8 kb IFN-α, not the 1.0 kb IFN-α, was the major species of IFN-α mRNA present in the poly IC/LC–activated monocytes. Because of the relaxed hybridization conditions used and because there are many IFN-α genes that may vary in their homology to the IFN-α cDNA we used (29), it is not certain if this is a true reflection of the quantities of different IFN-α mRNAs detected. It could be that the two IFN-α probes used here are more homologous to the 2.8 kb mRNA so that a stronger hybridization signal was detected. The 1.0 kb mRNAs may also represent one or more different IFN-α mRNAs of varying degrees of homology to the probes used in these experiments. A similar argument holds true for the 5.5 kb IFN-α mRNA. The mechanism for production of "nonsecretable" forms of IFN-α in MDP-stimulated human monocytes is not known; it is conceivable that the 2.8 kb IFN-α mRNA represents the transcriptional product of one of the IFN-α pseudo-genes.

The concept of the activated monocyte/macrophage has fascinated investigators worldwide. Detailed studies of the characteristics of animal monocyte/macrophages activated in vivo have produced extensive information regarding the functional characteristics (including monokine secretion) of the activated monocyte/macrophage (reviewed in 17–19); applications of this concept to the human monocyte situation have been anticipated. We propose here that the in vitro activated human monocyte might be composed of many monocyte activation subset states, each state centering around a particular monocyte function (or set of functions). We postulate that the regulatory mechanisms controlling each of these various activation states in vitro are similar to those operating in vivo. In this paper, we present the results of the first human monocyte studies defining the transcriptional synthetic basis for IFN-α production. Our studies complement recent reports of success in analyzing human monocyte C_2 mRNA metabolism (31).

Our present studies may have potential direct clinical applications. A number of disease states are thought to reflect (at least in part) monocyte defects (reviewed in 32). Some of these diseases might be related to aberrations in the production of IFN-α or other monokines. A specific clinical problem that we are currently investigating focuses on those cancer patients who become unresponsive to poly IC/LC immunotherapy in vivo (33, 34). Certain of these patients appear to demonstrate defects in IFN-α production. This defect might be traced to an abnormality in IFN-α-related macromolecular synthesis by the patient's mono-

cytes. We feel that the novel clinical and laboratory technologies presented in this report show the feasibility of further molecular biological studies in numerous activation and differentiation states of human monocytes, both in normal individuals and in patients.

Summary

Human monocytes were activated to secrete alpha interferon (IFN-α) by poly IC/LC but not by other monocyte activators, such as muramyl dipeptide (MDP). In contrast, monocytes were activated to secrete fibroblast growth factor (FGF) release by MDP but not by poly IC/LC. The amount of total RNA present in unactivated and activated human monocytes was similar. Using two ^{32}P-labeled cDNA probes (pLM001 and HuIFN-α₂) for human IFN-α genes in hybridization studies, we analyzed messenger RNA species from this gene family in activated human monocytes. After activation with poly IC/LC, two other mRNA species (2.8 and 5.5 kb) were detected in addition to the 1.0 kb mRNA normally associated with IFN-α secretion. Unexpectedly, monocytes activated with MDP also contained 2.8 kb IFN-α mRNA. There was associated with this 2.8 kb IFN-α mRNA, found in MDP-activated monocytes, appreciable levels of intracellular IFN-α activity in the absence of detectable secreted IFN-α. Thus the secretion of IFN-α in activated human monocytes can be correlated with the appearance of a 1.0 kb mRNA species after poly IC/LC exposure. Secretion appears to be defective in MDP-stimulated monocytes even though they contain active intracellular IFN-α apparently translated from the 2.8 kb mRNA.

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