Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8686–8690, December 1985
Medical Sciences

Administration of recombinant interferon γ to cancer patients enhances monocyte secretion of hydrogen peroxide

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Communicated by James G. Hirsch, August 12, 1985

ABSTRACT **Recombinant interferon γ (rIFN-γ) activates macrophage antimicrobial and antitumor functions and related metabolic processes, including secretion of reactive oxygen intermediates in mice and in cultured mouse and human macrophages. To look for similar actions in man, we monitored the H_zO_2 secretory capacity of monocytes from cancer patients receiving intravenous rIFN-γ at 0.1, 0.5, or 1.0 mg/m² of body area over 6 hr daily or over 1 hr on alternate days. Monocytes taken just before the first infusion served as controls and were comparable to normal donor monocytes in secretion of H_2O_2. Monocytes from 11 of the 13 subjects (85%) studied through 20 treatment cycles responded to rIFN-γ with elevation in H_2O_2 secretion in ≥67% of the tests conducted >1 hr after the start of treatment. Five of the five subjects tested had monocytes with diminished H_2O_2 secretory capacity when tested immediately after a 1-hr infusion of rIRN-γ, at which time the amount of adherent mononuclear cell protein recovered from the blood averaged only 24% of the control. At all other times tested (from 6 hr to 5 days after infusion) combined results for all subjects showed enhancement of H_2O_2 releasing capacity. Statistically significant mean increases ranged from 1.4- to 2.8-fold above the control and included the sets in which monocytes collected 24 hr following a single infusion were assayed the same day or the next. By the criterion of enhanced H_2O_2 secretory capacity, the ability of rIFN-γ to activate mononuclear phagocytes is manifest upon its administration to patients with advanced malignancy.**

Interferon γ (IFN-γ) is a glycoprotein secreted by lymphocytes that profoundly affects the physiology of mononuclear phagocytes. Monocytes, macrophages derived from monocytes in culture, or macrophages from tissues, when exposed *in vitro* to IFN-γ, show increased histocompatibility antigens and Fc receptors, secrete increased amounts of reactive oxygen intermediates and plasminogen activator, and more effectively kill tumor cells and microorganisms (reviewed in ref. 1). IFN-γ is far more potent than IFN-α or IFN-β in inducing most of these responses (e.g., refs. 2 and 3). With respect to enhancement of H_2O_2 secretion and the closely related antimicrobial activity of human (2, 4, 5) and murine (6, 7) macrophages, IFN-γ appears to be the predominant activating factor produced by polyclonal lymphocyte populations. The amounts of recombinant IFN-γ (rIFN-γ) sufficient to activate macrophage oxidative metabolism and antiprotozoal function are minute: picomolar concentrations (<1 antiviral unit/ml) with human (4) or mouse (6) cells *in vitro* and <100 units (<11 ng) after intraperitoneal injection in the mouse (6). Pretreatment of mice with rIFN-γ suppressed replication of *Listeria monocytogenes* in their spleens (8) and reduced mortality from *Toxoplasma gondii* (9).

These findings raise the possibility that rIFN-γ may activate mononuclear phagocytes in man. An opportunity to begin testing this hypothesis arose with the initiation of phase I trials of rIFN-γ in cancer patients (10) and the development of a quantitative assay for monocyte H_2O_2 secretion suitable for serial measuremens on a few milliliters of blood (11). The results provide evidence for the activation of human monocytes after the systemic administration of lymphokine.

MATERIALS AND METHODS

Study Population and Design. Adults with advanced malignancies refractory to conventional therapy received doses of 0.1, 0.5, or 1.0 mg/m² of body area rIFN-γ by continuous intravenous infusion in 5% (wt/vol) dextrose in water over a 6-hr period each day for 5 days each wk in 2-wk cycles. After a 2-wk rest, the treatment cycle was repeated. Monocyte function was studied immediately before the first infusion of each cycle, and at one or more of the following times: at the end of the first 6-hr infusion, or immediately before the infusions given on the 2nd, 3rd, 4th, or 5th day. In all, 98 assays (each with 3–10 replicates) were carried out on cells from 7 subjects. The same doses of rIFN-γ were administered in a second phase I trial over 1 hr on alternate days three times per wk. In this case, monocyte function was studied immediately prior to the first infusion, and at one or more of the following times: immediately after the first infusion, and on the next two mornings before any further infusion. In the second trial, 72 assays were performed in replicate on cells from 6 subjects. Each patient's pretreatment value served as a control. For comparison, blood was also obtained from untreated, normal adult donors.

rIFN-γ. Genentech, Inc., provided human rIFN-γ purified ≥95% from *Escherichia coli* with <0.5 ng of endotoxin/mg of protein and a specfic activity of ≈2 × 10⁷ antiviral units/mg of protein, as assayed by inhibition of encephalomyocarditis virus replication in A549 cells in comparison to the NIH IFN-γ standard. rIFN-γ was measured in serum by an ELISA using rabbit antibody to rIFN-γ.

Cell Preparation. Venous blood (5–10 ml) was collected in a heparinized Vacutainer tube or syringe. Mononuclear cells that were isolated on Ficoll/Hypaque (Pharmacia) as described (11) contained 1.8 ± 0.4% granulocytes (mean ± SEM, n = 44). Fewer than half of the granulocytes were adherent by the start of the first assay, as determined by inspection of stained culture wells. Mononuclear cells (≈20–30% with monocyte morphology) were cultured at 2–5 × 10⁵ cells per well in flat-bottomed, 96-well plastic tissue culture trays in 0.1 ml of RPMI 1640 medium with 25% (vol/vol) human serum, glutamine, and antibiotics as de-

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Abbreviations: IFN, interferon; r, recombinant.

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scribed (11). On average, 23 wells were cultured from each blood sample. Medium without cells was placed in control wells. Plates were incubated at 37°C in 5% CO_2/95% H_2O-saturated air for 2 hr (day 0 assay). For cultures to be assayed on days 1 or 2, the medium was aspirated through a 22-gauge needle after 2 hr of culture on day 0 and replaced with 0.1 ml fresh medium.

H_2O_2 Assay. The horseradish peroxidase-catalyzed oxidation of scopoletin by H_2O_2 was recorded by a fluorescent plate reader (Dynatech, Alexandria, VA) 0 and 60 min after addition of phorbol myristate acetate (PMA) (100 ng/ml) (Sigma) to adherent cells as described (11). Protein content in each well was measured (11) by using an absorbance plate reader (Bio-Tek, Burlington, VT). Specific release (nmol of H_2O_2 per mg of cell protein per 60 min) was calculated by microcomputer with corrections for the protein content and fluorescence changes in the cell-free wells (11).

RESULTS

Patient Characteristics and Baseline Studies. Seven men and six women were evaluated. In the 6-hr infusion study, 7 subjects ranging in age from 26 to 52 years (median age, 40) were studied during a total of 14 treatment cycles. Three had renal cell carcinoma and one each had medullary carcinoma of the thyroid, malignant melanoma, chronic myelogenous leukemia, and Hodgkin disease. In the 1-hr infusion study, 6 subjects ranging in age from 28 to 71 years (median age, 43) were studied during one treatment cycle each. Two had nodular poorly differentiated lymphoma and one each had medullary carcinoma of the thyroid, malignant melanoma, lymphocytic lymphoma, and Hodgkin disease. Before treatment with rIFN-γ, the ability of monocytes from these patients to release H_2O_2 was comparable to that of cells from normal blood donors (Table 1). The more rapid decline in monocyte H_2O_2 secretory capacity with time in culture in this study than has been reported (12) is characteristic of the microculture system employed here.

Serum Levels of rIFN-γ. An immunoassay with a sensitivity of 0.4 ng of rIFN-γ/ml detected no rIFN-γ in serum after infusion of 0.1 mg/m^2 of body area over a 6-hr period (Table 2). Values just above the limit of sensitivity were recorded when the same amount was infused over 1 hr. With doses of 0.5 or 1.0 mg/m^2 of body area, peak levels corresponding to 40–780 antiviral units/ml (assuming no loss of bioactivity) were detected at 0.5–6 hr, declining to baseline by 2.5–24 hr.

Effect of rIFN-γ Infusion on H_2O_2 Secretory Capacity. The effects of 6-hr infusions on a patient with metastatic melanoma are shown in Fig. 1. When monocytes were tested 2 hr after being placed in culture (the time allowed for them to adhere to the vessel), their capacity to secrete H_2O_2 in response to phorbol myristate acetate increased 41% over the pretreatment value by the end of the first infusion. Monocytes collected the next morning, before the second infusion of rIFN-γ had begun, released 1.8-fold more H_2O_2 than those collected prior to therapy. Results using cells taken ≈18 hr following the completion of the second, third, and fourth infusions showed 3.0-, 1.6-, and 2.7-fold enhancements. After 2 days without treatment, values returned to baseline (not shown). Less marked degrees of enhancement of H_2O_2 releasing capacity were seen when cells were cultured 1–2 days before assay and compared to pretreatment cells cultured for the same time.

Table 2. Serum levels of rIFN-γ

	Dose, mg/m^2 of body area	Peak level, ng of rIFN-γ/ml	Time to peak, hr	Time to return to UD, hr
6-hr infusion	0.1	UD (4)	NA	NA
	0.5	2.1–5.8 (3)	4–6 (3)	7–8 (3)
	1.0	10.4 (1)	6 (1)	24 (1)
1-hr infusion	0.1	0.4–0.6 (2)	0.5–1.5 (2)	2–24 (2)
	0.5	6.5–24.0 (2)	0.5 (2)	2.5–3.0 (2)
	1.0	8.6–39.0 (2)	0.5 (2)	2.5 (2)

The amount of rIFN-γ was determined in a subset of patients by using an ELISA; the limit of sensitivity was 0.4 ng of rIFN-γ/ml. Values are expressed as the range with the number of infusions in parentheses. UD, undetectable level; NA, not applicable.

The effects of a single 1-hr infusion are shown in Fig. 2. The capacity of monocytes to secrete H_2O_2 declined markedly when tested immediately after the infusion. By the next morning, however, H_2O_2 secretory capacity had rebounded to values much higher than the control. This elevation persisted for at least another day. Results were qualitatively similar whether the cells were tested 2, 24, or 48 hr after being placed in culture and compared to similarly cultured cells collected before the start of treatment.

Effects of Dose, Infusion Schedule, Number of Injections, and Day of Culture. To compare the findings in all 20 courses of treatment in all 13 subjects, results are expressed as the ratio of the posttreatment-H_2O_2-secretory capacity to the pretreatment value (Fig. 3).

Suppression of H_2O_2 releasing capacity was seen in two settings. First, in 5 of the 5 subjects whose cells were tested immediately after a 1-hr infusion, suppressed responses were noted in 13 of the 15 tests (5 subjects' cells assayed on days 0, 1, and 2 of the culture). Before the infusion, the yield of mononuclear leukocytes was 8.8 (± 1.4) × 10^5 cells/ml of blood from these subjects, whereas immediately following the 1-hr infusion, the yield declined to 3.9 (± 0.8) × 10^5 cells/ml. Adherent cell protein recovered 2 hr after placing the pretreatment cells in culture averaged 1.16 ± 0.12 μg of protein per 10^5 mononuclear cells plated. If on average 21% of these mononuclear cells were monocytes, the protein

Table 1. Pretreatment levels of monocyte H_2O_2 secretion with time in culture

Subjects	H_2O_2, nmol per mg per hr*		
	Day 0	Day 1	Day 2
Patients, 6-hr infusion	602 ± 60 (13)	255 ± 41 (13)	187 ± 29 (13)
Patients, 1-hr infusion	473 ± 52 (6)	171 ± 16 (6)	173 ± 20 (6)
All patients†	582 ± 45 (19)	229 ± 30 (19)	183 ± 21 (19)
Normal individuals	615 ± 62 (8)	190 ± 44 (7)	107 ± 26 (7)
P‡	>0.5	0.2–0.5	0.05–0.10

*Data are expressed as mean ± SEM of the number of experiments in parentheses.
†Weighted mean of values for patients from both groups.
‡Two-tailed t test for independent means comparing values for patients from both groups with values for normal individuals.

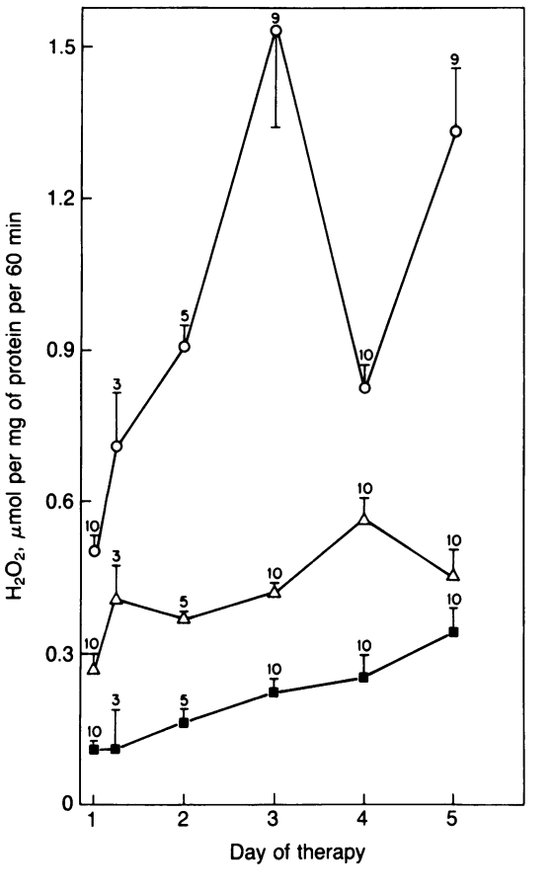

FIG. 1. Effect of daily 6-hr infusions of rIFN-γ on H_2O_2-releasing capacity of monocytes from a 40-year-old female with metastatic melanoma. Values for day 1 of therapy were from cells collected immediately before treatment. The next set of data points are from cells collected immediately after a 6-hr intravenous infusion of 0.5 mg/m² of body area (1.5×10^7 units) of rIFN-γ. On each of the following days, blood was drawn prior to infusion of rIFN-γ at the same dose. Cells were tested after 2 hr (open circles), 1 day (open triangles), or 2 days (solid squares) in culture. Means ± SEM for the number of replicates are indicated above each point.

content would correspond to the value of 5.5 μg of protein per 10^5 monocytes determined by another method (13). In contrast, with the immediate postinfusion samples, only 0.62 ± 0.05 μg of adherent cell protein were recovered per 10^5 mononuclear cells cultured. The combined effect of these changes [(3.9/8.8) × (0.62/1.16)] was that only about 24% as much adherent cell protein was recovered per ml of blood drawn immediately after a 1-hr infusion as per ml of blood drawn immediately before.

Second, monocytes from a 26-year-old male with stage IIIB Hodgkin disease and a 43-year-old male with metastatic renal cell carcinoma that were studied during several cycles of the 6-hr infusion protocol displayed diminished H_2O_2 releasing capacity in 14 of the 28 posttreatment tests. These two subjects account for 14 of the 17 solid symbols below the lines of unity in Fig. 3.

Excluding the situations described above, 52 of the 60 posttreatment tests (87%) showed enhanced H_2O_2 release. Overall, 11 of the 13 subjects (85%) responded to rIFN-γ infusion by the criterion that their monocytes secreted more H_2O_2 per mg of cell protein than in pretreatment tests, in ≥67% of tests performed >1 hr after the start of infusion.

There was no evidence for dose-dependence of the effects over the range studied. Therefore, to analyze overall responses, data at each time point were pooled for the three doses (Table 3). Including all subjects, enhancement H_2O_2 secretory capacity by rIFN-γ attained statistical significance for freshly tested monocytes collected 1 day after a 1-hr infusion and both 6 hr and 1 day after start of a 6-hr infusion. By the 3rd day of either protocol, results were statistically significant within experiments with some individuals but not for the whole group.

Effects of rIFN-γ Infusion on Monocyte Morphology. In four of the subjects, rIFN-γ infusion led to marked increases in the apparent diameter and degree of vacuolization of monocytes in cytocentrifuge preparations. These changes were clear-cut by 24 hr after the initiation of treatment, reversible on its cessation, and reinduced by its resumption (Fig. 4).

DISCUSSION

Eighty-five percent (11 of the 13) of patients with advanced maligancies responded to intravenous infusion of rIFN-γ with an increase in the capacity of their blood monocytes to secrete H_2O_2. Blood monocytes, in contrast to tissue macrophages, normally secrete abundant H_2O_2 (12) and, thus, are probably a suboptimal population for these studies. We studied monocytes because serial samples of tissue macrophages were not available. The enhancement of H_2O_2 releasing capacity seen here with monocytes may be a stringent test of the action of rIFN-γ on human mononuclear phagocytes *in vivo*. However, the important question remains open, whether tissue macrophages respond to rIFN-γ in a similar way. This seems likely, as human macrophages are responsive to rIFN-γ *in vitro*, not only when they are obtained by culture

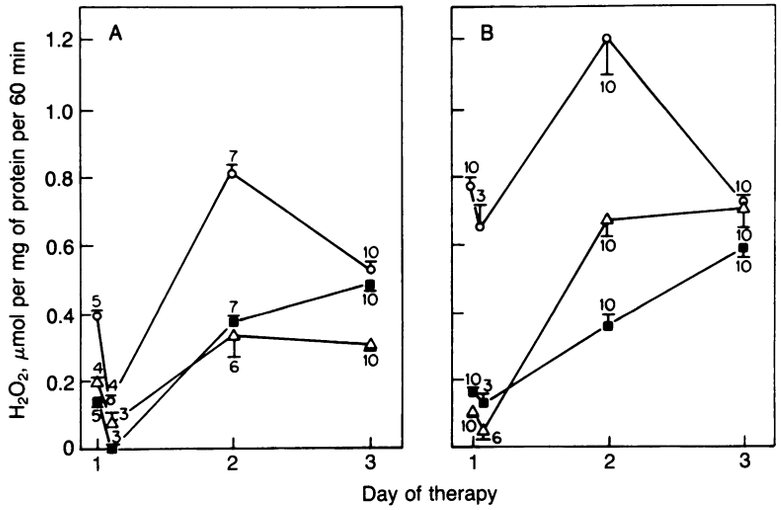

FIG. 2. Effect of a single 1-hr infusion of rIFN-γ on monocyte H_2O_2-releasing capacity. (*A*) Results for a 37-year-old female with metastatic melanoma. Values for day 1 of therapy were from cells collected immediately before treatment. The next set of data points are from cells collected immediately after a 1-hr intravenous infusion of 0.5 mg of rIFN-γ/m² of body area. Cells were collected on each of the next two mornings (days 2 and 3) without further administration of rIFN-γ. (*B*) Results for a 28-year-old female with stage IIIB Hodgkin disease who received 1 mg of rIFN-γ/m² of body area with samples taken as in *A*. Symbols are as in Fig. 1.

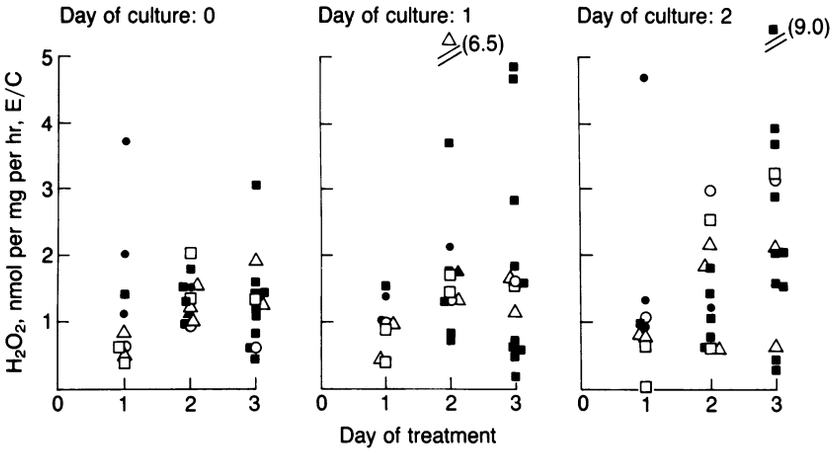

FIG. 3. Effect of rIFN-γ infusion on H_2O_2-secretory capacity of monocytes from each subject. Posttreatment (experimental, E) values are expressed as the fold increase (>1) or decrease (<1) relative to pretreatment (control, C) values obtained with the same subject's monocytes cultured for the same period of time prior to assay. Day 1 of treatment refers to cells collected immediately after the first infusion. Day 2 of treatment refers to cells collected the next day before any further treatment. Day 3 of treatment refers to cells collected on the 3rd day before that day's treatment. In the 1-hr study, the infusion on day 1 was the only one given during the period shown. In the 6-hr study, two infusions were given during the period shown: on day 1 and on day 2. Open symbols, 1-hr infusion treatment; solid symbols, 6-hr infusion treatment. Doses: 0.1 mg/m² of body area, circles; 0.5 mg/m² of body area, squares; 1.0 mg/m² of body area, triangles.

of blood monocytes (4, 5) but also when lavaged from pulmonary alveoli (14), and because an intravenous injection of rIFN-γ does activate peritoneal (6) and splenic (8) macrophages in the mouse.

There are few reported examples of increased mononuclear phagocyte function after administration of cytokines to man, and to our knowledge, none involving IFN-γ. Injection of IFN-α failed to augment monocyte superoxide releasing capacity (15) or the phagocytosis (16) or killing (17) of yeasts, although Fc receptor expression was increased (18). In two studies, mononuclear leukocytes from subjects treated with IFN-α were better able to inhibit [^{3}H]thymidine uptake by a murine tumor cell line than were pretreatment leukocytes (19, 20). Neither study used monocytes depleted of natural killer cells nor confirmed inhibition of tumor cell growth. High ratios of effector to target cells and of cells to volume of culture fluid, combined with the ability of mononuclear phagocytes to secrete thymidine, add to the difficulty of drawing firm conclusions. Other investigations revealed no consistent enhancement of monocyte cytotoxic activity after injection of IFN-α (17, 21). Thus, the relatively limited ability of IFN-α to activate macrophages *in vitro* (2, 3) appears to be borne out for monocytes *in vivo*.

By the criterion of enhanced H_2O_2 secretory capacity, the findings described in this report appear to be the first to show that human mononuclear phagocytes can be activated *in vivo* by a secretory product of immunologically activated lymphocytes. This interpetation rests on the close correlation of H_2O_2 secretory capacity with the antimicrobial and antitumor activity of mononuclear phagocytes, in part reflecting the direct involvement of reactive oxygen intermediates in cytotoxicity by macrophages in several experimental settings (6, 22, 23). However, mononuclear phagocytes are equipped with additional cytotoxic mechanisms (22, 24–26), and these may also be enhanced by IFN-γ (26). It remains to be shown whether macrophage antimicrobial or antitumor activity is actually induced by rIFN-γ in man, and if so, which cytotoxic mechanisms are operative.

One of the primary purposes of the experiment in which these patients participated was to define a maximal tolerated dose of rIFN-γ (10). The present findings raise other questions. How little rIFN-γ in man is required to activate monocytes? Do doses ≤0.1 mg/m² of body area, which appeared to activate monocytes with few detectable side effects (10), have as much antitumor or antimicrobial efficacy as higher, less well tolerated doses? Is monocyte H_2O_2 secretory capacity a useful indicator of the desired effects of rIFN-γ when testing variables such as the route and interval of administration?

A single intravenous injection of 0.1 mg/m² of body area rIFN-γ activated monocytes within 6–24 hr, and although waning, this effect persisted in some subjects over a period of 3 days. The onset of activation in both mouse (6) and man thus appears to be faster after administration of rIFN-γ *in vivo* than *in vitro* (4–6). The reasons for this are unknown.

The suppression of monocyte H_2O_2-releasing capacity immediately following a 1-hr but not a 6-hr infusion may reflect schedule-dependent toxicity to monocytes. Alternatively, the transiently decreased yield of mononuclear leukocytes per ml of blood and of the yield of adherent cell protein per 10^5 mononuclear leukocytes suggest that typical monocytes may have been redistributed in the vasculature

Table 3. Effect of intravenous rIFN-γ on human monocyte H_2O_2-releasing capacity

Infusion schedule	Day of cycle	Relative amount of H_2O_2 released on day of culture		
		0	1	2
1 hr, alternate days	1*	0.59 ± 0.07 (5) [0.03]	0.73 ± 0.12 (5)	0.68 ± 0.16 (5) [0.06]
	2†	1.36 ± 0.15 (6) [0.05]	2.46 ± 0.90 (5) [0.03]	1.80 ± 0.38 (6)
	3‡	1.30 ± 0.24 (4)	1.49 ± 0.10 (4) [0.06]	2.29 ± 0.52 (4)
6 hr, daily	1*	2.07 ± 0.50 (4) [0.06]	1.31 ± 0.12 (3)	1.99 ± 0.79 (4)
	2‡	1.62 ± 0.43 (6) [0.03]	1.75 ± 0.35 (7) [0.02]	1.17 ± 0.16 (6)
	3§	1.32 ± 0.24 (9)	1.85 ± 0.55 (9)	2.75 ± 0.79 (9) [0.001]

H_2O_2 (μmol per mg of protein per hr) was measured and the results were pooled for all three dose levels for all subjects. Each posttreatment value was compared to the same subject's pretreatment value for cells cultured the same length of time, using Wilcoxon's rank sum test for paired means. Data are the mean of the fraction of pretreatment values ± SEM. The number of patients participating is in parentheses. $P \leq 0.06$ are in brackets.

*Cells were collected immediately after the first infusion.

†Cells were collected 24 hr after the first infusion.

‡Cells were collected immediately before the second infusion.

§Cells were collected immediately before the third infusion.

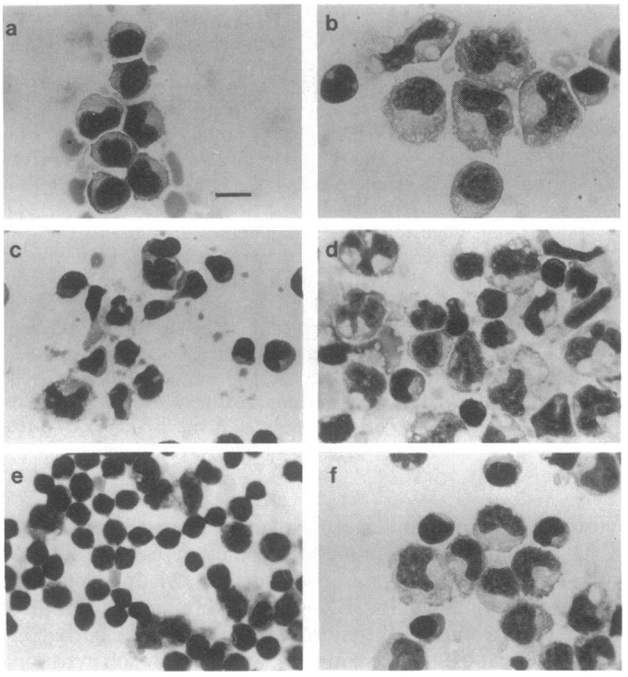

FIG. 4. Photomicrographs of Diff-Quik stained cytocentrifuge preparations of mononuclear leukocyte suspensions. (*a–d*) Cells from the same subject as in Fig. 1. (*a*) Cells before first infusion of rIFN-γ. (*b*) Cells before second infusion. (*c*) Cells before first infusion in the second cycle (1 month after *a*, 2 weeks after last treatment). (*d*) Cells before second infusion in the second cycle. (*e*) Pretreatment cells from a 42-year-old woman with nodular poorly differentiated lymphoma. (*f*) Cells from the latter patient 2 days following a 1-hr infusion of rIFN-γ (0.5 mg/m^2 of body area). Bar = 10 μm. Cytocentrifugation, staining, and photomicroscopy conditions were the same in each case.

during the 1-hr infusion, perhaps by margination. Yields of total and adherent mononuclear cells at all other times studied after administration of rIFN-γ did not differ from pretreatment values (not shown). This suggests that the cells collected from the patients at such times were probably as representative of monocytes in the bloodstream as were the cells obtained prior to treatment.

It is possible that administration of rIFN-γ may favor the circulation of a more or less mature subpopulation of monocytes than normal and that this subset may be more competent to secrete H_2O_2 than that usually circulating. On the other hand, rIFN-γ can augment H_2O_2 secretory capacity in cultured mononuclear phagocytes without a change in cell number (2, 4, 6), and thus can alter the metabolism of individual cells. Similar considerations apply to the apparent enlargement and vacuolization of posttreatment monocytes obtained from about one-third of the subjects.

In conclusion, the ability of rIFN-γ to activate mononuclear phagocytes, one of its most prominent and potent immuno-modulatory effects *in vitro*, appears to be manifest upon its administration to patients with advanced malignant disease. This invites further clinical study in settings in which there is a rationale for activating macrophages, such as infection by certain intracellular pathogens.

This work was supported by Grants CA22090 and CA33049 from the National Institutes of Health and by Genentech, Inc. C.F.N. is a scholar of the Rita Allen Foundation.

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