Activation of Monocytes by Interferon-Gamma Has No Effect on the Level or Affinity of the Nicotinamide Adenine Dinucleotide-Phosphate Oxidase and on Agonist-dependent Superoxide Formation

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Abstract

Human monocytes purified by elutriation were cultured for 3 d in Teflon bags with or without human recombinant interferon-gamma (rIFNγ). The cells were then collected and used in suspension to determine the rate of stimulus-dependent superoxide or hydrogen peroxide formation as a measure of the NADPH-oxidase. The treatment with IFNγ increased this rate two- to threefold when phorbol myristate acetate (PMA) was used as the stimulus. By contrast, no IFNγ-dependent increase in superoxide production was observed when the cells were stimulated with different concentrations of the receptor agonist *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) alone or in combination with another receptor agonist, platelet-activating factor (PAF). At optimum concentrations, f-Met-Leu-Phe elicited rates of superoxide formation that could not be exceeded under other stimulatory conditions including PMA after treatment with IFNγ. It thus appears that f-Met-Leu-Phe can lead to maximum activation of the NADPH-oxidase, and that this response is not influenced by IFNγ. Treatment with IFNγ also failed to affect the affinity of PMA- or f-Met-Leu-Phe–stimulated oxidase for NADPH, the K_m values being 30 to 40 μM under all conditions. IFNγ did not alter the cellular levels of cytochrome b_{558}, as measured by low-temperature spectroscopy, and protein kinase C, as measured by [^{3}H]phorbol dibutyrate binding, and did not appreciably influence the stimulus-dependent increase of cytosolic free calcium.

These results indicate that activation of human mononuclear phagocytes by IFNγ does not affect the level and the kinetic properties of NADPH-oxidase or its activation by receptor agonists. They confirm, however, that IFNγ enhances the respiratory burst response to PMA.

Introduction

It is well established that the macrophage-activating factor produced by stimulated T lymphocytes corresponds to interferon-gamma (IFNγ)[1] (1–5). Human mononuclear phagocytes cultured in the presence of IFNγ produce higher amounts of hydrogen peroxide after stimulation with phorbol myristate acetate (PMA) (1, 6). This increased metabolic capacity has been related to the enhanced antimicrobial and cytotoxic activity of IFNγ-treated macrophages (2, 7, 8), and has been considered a biochemical correlate of macrophage activation (9). It was shown that human macrophages have to be treated with IFNγ for a few days to develop maximum responsiveness to PMA, suggesting the involvement of an inductive process (1, 6). The higher production of hydrogen peroxide has been related to an increased affinity of the respiratory burst oxidase for its substrate, NADPH (10). Enhanced NADPH-oxidase activity and a decreased K_m for NADPH have been reported earlier for murine peritoneal macrophages activated by various in vivo treatments (11–13).

All the above studies were performed with adherent cells, and the amounts of hydrogen peroxide released were determined over time periods of 30–120 min. In the present investigation we have attempted to gain more direct information on the NADPH-oxidase activity using kinetic measurements. Human monocytes purified by elutriation were activated by culturing in the presence of recombinant IFNγ (rIFNγ) in Teflon bags, and the rates of superoxide or hydrogen peroxide production after stimulation with PMA or the receptor agonists *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) and platelet-activating factor (PAF) were recorded using single-cell suspensions. Our results confirm the earlier findings with PMA as the stimulus (1, 6, 10), but show that IFNγ treatment does not enhance the respiratory burst response of human macrophages to f-Met-Leu-Phe alone or in combination with PAF and does not alter the level of the oxidase nor its affinity for NADPH.

Methods

Materials. f-Met-Leu-Phe and PAF were obtained from Bachem AG, Bubendorf, Switzerland; Lympho-paque from Nyegaard, Oslo, Norway; BSA, leupeptin and quin-2/AM from Fluka AG, Buchs, Switzerland; cytochrome *c* (type III), phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) from Sigma Chemical Co., St. Louis, MO; horseradish peroxidase (405 U/mg) from SERVA GmbH, Heidelberg, FRG; [^{3}H]PDBu from New England Nuclear, Zürich, Switzerland; DME from Gibco AG, Basel, Switzerland. rIFNγ with a specific activity of 9.5×10^6 U/mg was obtained from Hoffmann-La Roche Ltd., Basel, Switzerland. SOD was kindly provided by Dr. L. Flohé, Grünenthal AG, Aachen, FRG. All other chemicals were of analytical grade.

Monocytes were purified from buffy coats of citrated blood from single donors by centrifugation on Lympho-paque. The mononuclear layer was washed three times with phosphate-buffered saline contain-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; HBSS/BSA, Hanks' balanced salt solution containing 2.5 mg/ml bovine serum albumin; IFNγ and rIFNγ, (recombinant) interferon-gamma; PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; quin-2 and quin2/AM, 2-[(2-amino-5-methylphenoxyl)-methyl]-6-methoxy-8-aminoquinoline-*N,N,N',N'*tetraacetic acid and teraacetoxymethylester, respectively.

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ing 5% BSA and 0.5 mM EDTA, and then subjected to centrifugal elutriation (14). Monocyte fractions of purity > 90% were pooled, washed once with DME containing 4.5 g/liter D-glucose and finally resuspended at 10^6 cells/ml in DME supplemented with 13% acid-treated human AB plasma (14).

Teflon bag cultures. Monocytes (10^6 cells/ml) were cultured for 3 d as described by Andreesen et al. (15) with some modifications. Monocyte suspensions in media containing the appropriate amount of rIFNγ were poured into ultraviolet sterilized Teflon bags which were sealed with adhesive tape. After 3 d at 37°C in 5% CO_2/95% air, the bags were rubbed with the fingers and opened by cutting an edge off. The cells were collected, cooled on ice for 5 min, centrifuged at 250 *g* for 10 min at 2°C and resuspended (6×10^6 cells/ml) in cold HBSS/BSA with half the concentration (0.5 mM) of Ca^{2+} and Mg^{2+}.

Adherent cultures. Monocytes (0.1×10^6 cells/100 μl) were distributed in flat-bottom 96-well culture plates and diluted with 100 μl DME supplemented with acid-treated human AB plasma and the appropriate amounts of rIFNγ.

Superoxide formation was measured at 37°C as the SOD sensitive reduction of ferricytochrome *c* (16). The assay mixture (800 μl) consisted of 0.75×10^6 cells per ml HBSS/BSA containing 85 μM cytochrome *c*. Absorbance changes were recorded in a diode array spectrophotometer (8451A; Hewlett-Packard, Palo Alto, CA) equipped with a seven-place cuvette exchanger. Due to the instrument's spectral resolution of 2 nm per diode and the high cytochrome *c* concentration needed, a deflection from linearity of the absorbance increase at 550 nm (the very narrow alpha band) was observed above 50% cytochrome *c* reduction (Fig. 1). The measurements were thus based on the absorbance increase of the broad beta band (peak at 520 nm) with respect to a reference obtained as the mean of three isosbestic points. The absorbance increase ΔA, calculated as: $\Delta A = 1/5\,(A_{514} + A_{516} + A_{518} + A_{520} + A_{522}) - 1/3\,(A_{504} + A_{526} + A_{542})$ was found to be linear over the whole transition from ferri- to ferrocytochrome *c* and a mean difference extinction coefficient of $\epsilon = 5.13\ mM^{-1}\ cm^{-1}$ was used for calculations. Redox titration spectra of cytochrome *c* are shown in Fig. 1. The integration of the absorbance values and the use of the mean of three isosbestic points as the reference markedly reduced the noise of the measurements in cell suspensions. The maximum rates of ferrocytochrome *c* formation were obtained from the first derivative of the recorded progress curves, and the values for superoxide formation were calculated.

Hydrogen peroxide formation was measured according to Hyslop and Sklar (17). The assay mixture consisted of 0.75×10^6 cells/ml HBSS/BSA, 25 U/ml horseradish peroxidase, 50 μg/ml SOD and 1.15 mM *p*-hydroxyphenyl acetate. Fluorescence changes were recorded at

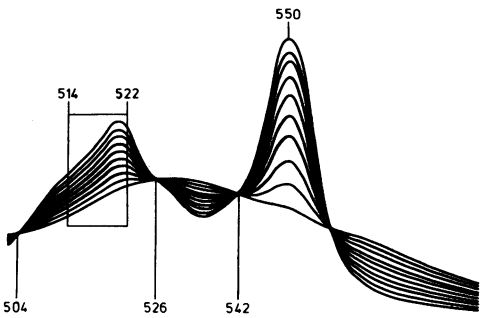

Figure 1. Redox titration of cytochrome *c*. Solutions of ferricytochrome *c* (5% reduced) and ferrocytochrome *c* (5% oxidized), both 85 μM in 50 mM sodium phosphate buffer, pH 7.4, were mixed and the spectra were recorded. Starting with 5% (bottom trace at 550 nm), the concentration of reduced cytochrome *c* was increased by steps of 10% and the spectra were recorded (top trace 95% reduced). The beta band region (514 nm to 522 nm) and the three isosbestic points (504, 526, and 542 nm) used to calculate the cytochrome *c* reduction are indicated.

37°C in a spectrometer (LS-5; Perkin-Elmer Corp., Norwalk, CT) (exitation: 323 nm, emission: 400 nm). The signals were quantified with standard concentrations of hydrogen peroxide ($\epsilon_{240\ nm} = 40\ M^{-1}\ cm^{-1}$). Hydrogen peroxide accumulation of adherent cells was measured as described by Ruch et al. (18).

Stimulation. For the measurement of superoxide and hydrogen peroxide production of cell suspensions f-Met-Leu-Phe and PMA were dissolved in HBSS/BSA containing sodium azide to give a final concentration of 1 mM during the assay. The presence of sodium azide was found to be critical for obtaining complete recoveries of hydrogen peroxide and since it was slightly inhibitory when given to the cells in advance, it was added together with the stimulus. In all other experiments the simuli were added in HBSS/BSA without azide.

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Co. Inc., Yellow Springs, OH). The cells (3×10^6 in 900 μl HBSS/BSA) were prewarmed for 5 min at 37°C. Mitochondrial respiration was then inhibited with 1.1 mM sodium azide yielding a flat baseline, and the respiratory burst was elicited after 1 min by the addition of the appropriate stimulus. Calculation: air saturated HBSS/BSA was assumed to contain 220 μM O_2 and zero oxygen tension was obtained by adding sodium dithionite.

NADPH-oxidase, measurement of the K_m for NADPH. Samples of 1.6 ml (2.2×10^6 mononuclear phagocytes per ml HBSS/BSA) containing 85 μM cytochrome *c* were stimulated at 25°C with 500 nM f-Met-Leu-Phe or 300 nM PMA in a continuously stirred cuvette, and ferrocytochrome *c* formation was followed spectrophotometrically (see above). At maximum rate of superoxide formation (15 or 60 sec. after stimulation), the cells were lysed with 0.063% sodium deoxycholate and 1.25 mM EGTA, leading to a stop of superoxide production within a few seconds. The reaction was restarted 15 s later by the addition of NADPH (5 to 200 μM). The initial rates of superoxide formation were recorded and used to calculate the K_m value by Eadie-Hofstee analysis.

Low temperature spectroscopy was performed at 77°K in a custom built single-beam spectrophotometer specially designed for highly scattering material according to Butler (19) with some modifications (Thelen, M., and V. von Tscharner, unpublished).

Calcium. Cytosolic free calcium ($[Ca^{2+}]_i$) changes were measured with quin-2 according to von Tscharner et al. (20). Briefly, monocytes were diluted to 4×10^6 cells/ml with 130 mM NaCl, 4.6 mM KCl, 5 mM $NaHCO_3$, 5.6 mM D-glucose, 20 mM Na-HEPES, pH 7.4, and loaded for 15 min at 37°C with 0.6 nmol/10^6 cells quin-2/AM (3 μl/ml of 0.8 mM quin-2/AM in acetone). The cells were then centrifuged for 8 min at 160 *g*, resuspended in the above medium containing in addition 1 mM $CaCl_2$, and stimulus-dependent $[Ca^{2+}]_i$ changes were recorded fluorimetrically (20).

Phorbol ester binding. Aliquots of 600 μl of the mononuclear phagocytes taken directly from the Teflon bags were distributed in 5-ml glass test tubes. The cells were washed twice with 600 μl HBSS/BSA (150 *g* for 10 min) and incubated for 30 min at room temperature in 150 μl HBSS/BSA containing 5 to 100 nM [^{3}H]PDBu and 6 μg leupeptin. Unspecific binding was estimated in the presence of 10 μM unlabeled PDBu (21). The cells were diluted with 1 ml ice cold PBS, centrifuged, and washed two times with the same buffer and lysed with 200 μl of 2 M LiCl containing 0.01% digitonin. One part of the lysates was used for scintillation counting, and the other for DNA determination (see below).

DNA measurements. The DNA content of cell suspensions and monolayers was determined using the method of Kapuscinski and Skoczylas (22) after cell lysis in 2 M LiCl containing 0.01% digitonin.

Results

Culture conditions. Our studies of the effect of IFNγ on the respiratory burst required the use of homogeneous mononuclear phagocyte suspensions since rapid kinetic measurements

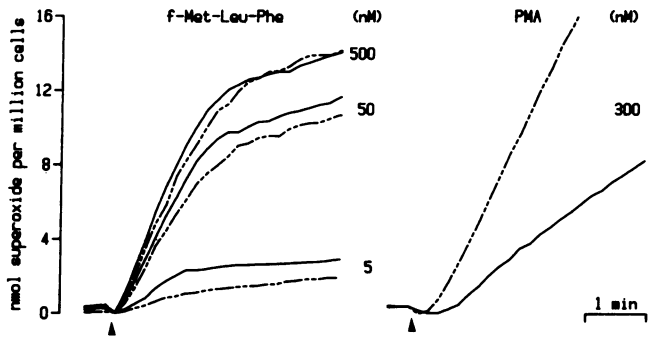

Figure 2. Superoxide formation by human mononuclear phagocytes. Monocytes (0.75×10^6/ml) were cultured for 3 d in the presence (*broken line*) or absence (*solid line*) of 60 U/ml rIFNγ and then stimulated (*arrowheads*) with f-Met-Leu-Phe or PMA at the concentrations indicated. Tracings are computer averages of duplicate continuous recordings of cytochrome *c* reduction. Similar results were obtained with six different monocyte preparations.

could not be done accurately on adherent cells. As the conditions for activation had been worked out on adherent cultures (1, 6, 9), we first attempted to suspend adherent cells by mechanical or proteolytic treatments. The results were unsatisfactory because of cell damage and, in particular, of different recoveries from IFNγ-treated and nontreated cultures. The possibility was therefore explored to culture and treat the monocytes in Teflon bags (15). Comparative experiments showed that both culture systems are suitable to study the effects of IFNγ. A 3-d exposure to IFNγ (60–100 U/10^6 cells) enhanced the PMA-elicited hydrogen peroxide production by adherent cells from 2.13±0.58 to a maximum of 5.14±0.51 nmol per 0.1×10^6 cells in 2 h (three cell preparations) and the maximum rate of PMA-dependent hydrogen peroxide production by cells cultured in Teflon bags from 2.94±0.23 to 5.88±0.39 nmol/min per 10^6 cells (six cell preparations).

Using adherent cells, the effect of IFNγ was similar when the treatment was started on freshly prepared monocytes (day 0) or after a culture period of 3 d (6). All treatments in Teflon bags were therefore started on day 0. Routinely, DME was supplemented with 13% acid-treated human AB plasma from single donors. This plasma was compared with autologous acid-treated plasma at two concentrations (13 and 25%). Both in Teflon bags and plastic dishes, these variations were without effect on the respiratory burst activity of the cells.

Effect of IFNγ pretreatment on the rate of superoxide production. Continuous recordings of the superoxide released by the cells are shown in Fig. 2. The response to f-Met-Leu-Phe was almost immediate and leveled off after 2–3 min while the response to PMA was somewhat delayed and of much longer duration. In both cases, the rate of superoxide production depended on the stimulus concentration (as shown for f-Met-Leu-Phe). When maximum effective concentrations (i.e., 500 nM f-Met-Leu-Phe or 300 nM PMA) were applied to control cells, the rate of superoxide production elicited by the chemotactic peptide was much higher than that elicited by PMA. Similar curves were obtained when hydrogen peroxide was measured instead of superoxide. In parallel recordings, using f-Met-Leu-Phe or PMA as stimulus, a ratio of ~ 2 mol of superoxide per mol of hydrogen peroxide was obtained, indicating that superoxide was the primary product of the NADPH-oxidase and that hydrogen peroxide resulted from its dismutation (Table I).

Table I. Stoichiometry of Superoxide and Hydrogen Peroxide Formation by Human Monocytes

		O_2^-	H_2O_2	O_2^-/H_2O_2	*n*
f-Met-Leu-Phe	500 nM	5.51±0.25	2.66±0.2	2.06±0.18	6
PMA	300 nM	2.6±0.45	1.37±0.12	1.92±0.37	6

Monocytes were cultured for 3 d and stimulated with maximum effective concentrations of f-Met-Leu-Phe or PMA. The rates of superoxide and hydrogen peroxide formation (nmol per min per 10^6 cells) were determined in parallel with each cell preparation.

Fig. 2 also shows the results obtained with cells that were cultured for 3 d with IFNγ. The rate of superoxide production induced by PMA was increased two- to threefold over that of the untreated control. By contrast IFNγ did not change appreciably the superoxide production elicited by 50 or 500 nM f-Met-Leu-Phe. Treated cells actually tended to weaker responsiveness to the chemotactic peptide, a finding that was clearly evident when the receptor agonist was used at low concentrations (e.g., 5 nM), but which was not investigated further. It is important to note that the maximum activity of the NADPH-oxidase of IFNγ-treated cells stimulated with PMA was about equal to that elicited by f-Met-Leu-Phe (both in control and IFNγ-treated cells). Equal maximum rates of product formation were also obtained with either stimulus when lower numbers of cells were used and when hydrogen peroxide was determined, excluding the possibility of an experimental artefact due to limitations of the superoxide detection system (data not shown). The different effects of IFNγ treatment on the respiratory burst response to f-Met-Leu-Phe or PMA at optimum concentrations are further documented in Fig. 3. The activity of the NADPH-oxidase stimulated with f-Met-Leu-Phe was already close to its maximum at day 0, and remained practically unaffected by IFNγ. With PMA as the stimulus, however, IFNγ treatment was necessary for maximum responsiveness. Following culture with IFNγ concentrations above 30 U/ml superoxide production was virtually identical with either stimulus.

Similar sets of data were obtained when respiratory burst-dependent oxygen consumption was measured. Fig. 4 shows that IFNγ treatment enhanced the rate of oxygen consumption

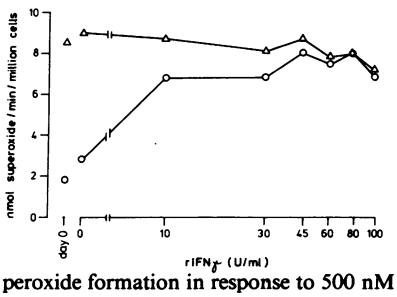

Figure 3. Effect of the IFNγ concentration on the rate of superoxide formation by human mononuclear phagocytes. Monocytes were cultured for 3 d with the indicated concentrations of rIFNγ, and the maximum rate of superoxide formation in response to 500 nM f-Met-Leu-Phe (△) or 300 nM PMA (○) was determined (see Fig. 2). The activity of freshly eluted monocytes from the same donor in the absence of rIFNγ is also indicated (day 0). Means of duplicate measurements. Similar results were obtained in six additional experiments.

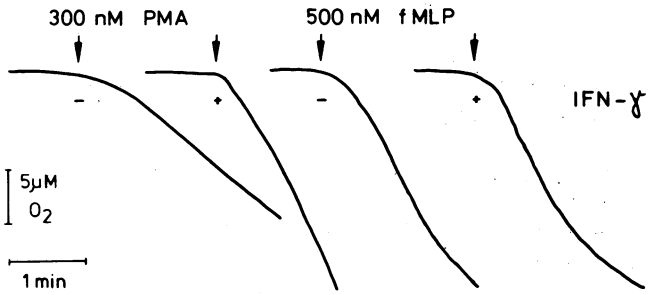

Figure 4. Effect of IFNγ on respiratory burst dependent oxygen consumption by human mononuclear phagocytes. Monocytes were cultured for 3 d in the presence (+) or absence (−) of 60 U/ml rIFNγ and then stimulated with 300 nm PMA or 500 nm f-Met-Leu-Phe (*arrowheads*).

following stimulation with PMA but not with f-Met-Leu-Phe. As in the case for superoxide production, maximum rates of oxygen consumption were obtained in control cells stimulated with f-Met-Leu-Phe alone.

In view of the lack of effect of IFN*g* on the rate of superoxide production elicited by f-Met-Leu-Phe, further experiments were done with combinations of f-Met-Leu-Phe and PAF which are known to act synergistically on the respiratory burst of neutrophils (23). Fig. 5 *a* shows that such a synergism was also observed in mononuclear phagocytes. A preexposure (30 s) to 10 nM PAF, which by itself induced only a minimal response, markedly increased both the rate and the amount of superoxide production in response to 5 or 50 nM f-Met-Leu-Phe. The effect of PAF was more pronounced at low f-Met-Leu-Phe concentrations, and superoxide formation was not further enhanced when PAF was combined with a maximum effective concentration of 500 nM f-Met-Leu-Phe (data not shown). A similar synergism between both agonists was also obtained in IFNγ-treated cells (Fig. 5 *b*). The tracings of superoxide production had the same course as those shown in Fig. 2. As in those experiments, IFNγ treatment did not enhance agonist-dependent superoxide production, and the maximum activity of the NADPH-oxidase obtained with f-Met-Leu-Phe alone could not be exceeded.

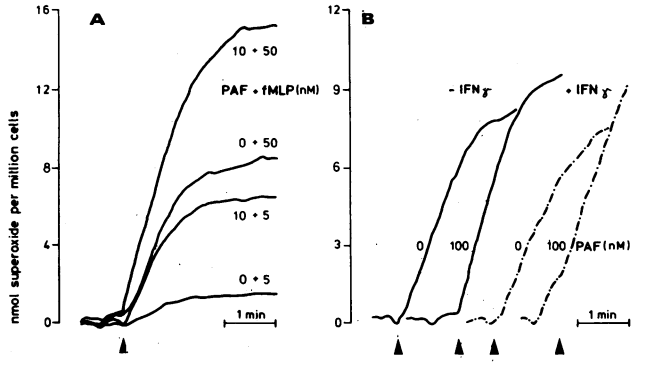

Figure 5. Effect of IFNγ on superoxide formation induced by PAF and f-Met-Leu-Phe. (*A*) Monocytes were cultured for 3 d and then stimulated with f-Met-Leu-Phe with or without preexposure (30 s) to 10 nM PAF. (*B*) Monocytes were cultured for 3 d in the presence (*broken lines*) or in the absence (*solid lines*) of 60 U/ml rIFNγ and then stimulated with 50 nM f-Met-Leu-Phe with or without preexposure (30 s) to 100 nM PAF. Tracings are computer averages of duplicate recordings representative for six different monocyte preparations.

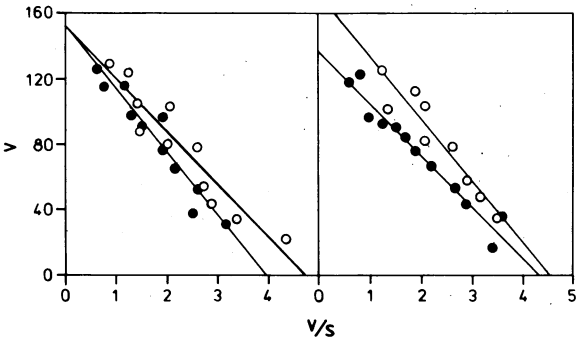

Figure 6. Effect of IFNγ on the K_m of the NADPH-oxidase for its substrate NADPH. Eadie-Hofstee plots. Monocytes were cultured for 3 d in the presence or absence of 60 U/ml IFNγ and then stimulated with 300 nM PMA (*left*) or 500 nM f-Met-Leu-Phe (*right*) at 25°C. After cell lysis NADPH-dependent oxidase activity was measured at NADPH concentrations between 5 and 200 μM. Linear regression analysis yielded K_m values of 38.4 μM (PMA) and 31.4 μM (f-Met-Leu-Phe) for IFNγ-treated cells (●) and of 31.9 μM (PMA) and 37.5 μM (f-Met-Leu-Phe) for control cells (○). v, pmol superoxide per min per μg DNA; s, NADPH (μM).

Effect of IFNγ on the K_m of the NADPH-oxidase for its substrate NADPH. The possibility was considered that superoxide formation elicited by f-Met-Leu-Phe and PMA may involve NADPH-oxidase pools differing in their substrate affinity, and that IFNγ treatment may decrease the K_m of the NADPH-oxidase stimulated with PMA. In lysates of resting mononuclear phagocytes, the respiratory burst could not be elicited by the addition of the stimuli or NADPH (not shown). However, when the cells were lysed after the respiratory burst had been induced with f-Met-Leu-Phe or PMA, superoxide formation could be reinitiated by the addition of NADPH. The affinity of the oxidase for NADPH was similar following stimulation with f-Met-Leu-Phe or PMA and was not affected by the IFNγ treatment as shown by a representative experiment in Fig. 6. In eight separate determinations, the K_m values varied between 30 and 40 μM NADPH. The maximum velocity values (V_{max}) showed a somewhat higher range of variation between different donors (130–210 pmol/min per μg DNA) but were equally unaffected by IFNγ treatment. The results suggest that superoxide production induced by f-Met-Leu-Phe and PMA depends on the same oxidase and that treatment with IFNγ does not alter the kinetic properties of the enzyme.

Effect of IFNγ on the cytochrome b_{558} level. The terminal component of the NADPH-oxidase, which transfers electrons to molecular oxygen, is a low-potential b-type cytochrome with a characteristic absorbance peak at 558 nm at 77°K (24, 25). The effect of IFNγ on the expression of this cytochrome was studied in three different cell preparations. The upper trace in Fig. 7 shows a representative low-temperature reduced minus oxidized spectrum of IFNγ-treated mononuclear phagocytes with a prominent absorbance peak at 558 nm and a shoulder at 556 nm which are typical for cytochrome b_{558}. The high resolution at 77°K permits to use the absorbance of mitochondrial cytochromes *c* and aa_3 for comparison with the content of cytochrome b_{558} even though other b-type cytochromes of the cells have their absorbance maxima in close proximity. The nearly horizontal lower trace in Fig. 7 represents the difference in absorbance of the reduced spectra of

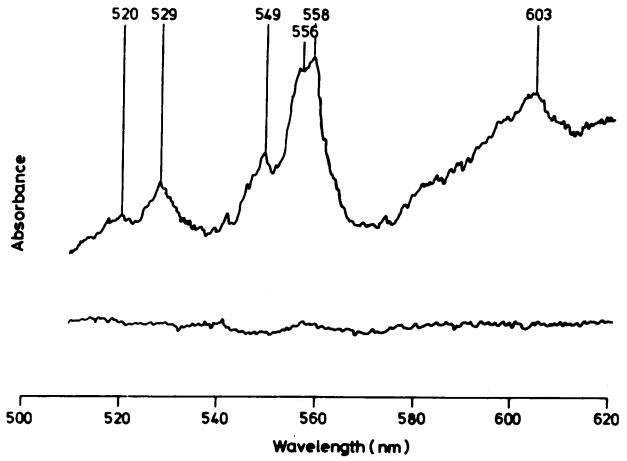

Figure 7. Low temperature absorbance-difference spectra of intact human mononuclear phagocytes. Spectra were recorded from 510 to 620 nm. (*Upper trace*) Reduced minus oxidized difference spectrum of monocytes cultured for 3 d in the presence of 80 U/ml rIFNγ. Two cuvettes containing each 40×10^6 cells in 450 μl were prepared, to one of them few grains of sodium dithionite were added and both cuvettes were then frozen in liquid nitrogen. The spectrum of the oxidized sample was stored in a scan recorder (model 4101; Princeton Applied Research, Princeton, NJ) and subtracted from that of the reduced one. (*Lower trace*) Difference spectrum of IFNγ treated minus nontreated monocytes. Monocytes were cultured for 3 d in the presence or absence of 80 U/ml rIFNγ, washed and brought to the same DNA content (~ 90×10^6 cells/ml) in 350 μl. Both samples were reduced with a few grains of sodium dithionite for 10 s and then immediately frozen in liquid nitrogen, and the spectra were recorded. The first spectrum was stored in the scan recorder and subtracted from the second. Vertical lines indicate absorbance peaks of the cytochromes aa_3 (603 nm), *b* (558 nm, 529 nm), and *c* (549 nm, 520 nm), respectively. The absorbance scale is arbitrary due to the unknown light path in the samples. The data are representative for three experiments made with different monocyte preparations.

IFNγ-treated and untreated cells, indicating that both populations of mononuclear phagocytes have the same cytochrome content, and suggesting that IFNγ did not induce the synthesis of cytochrome b_{558} or of an analogous cytochrome.

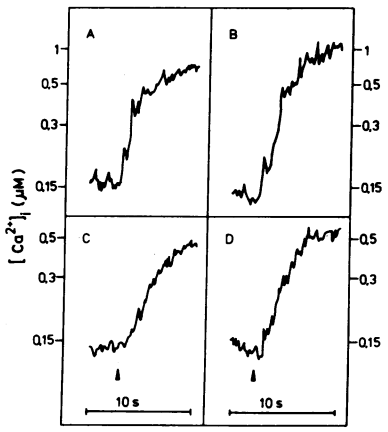

Figure 8. $[Ca^{2+}]_i$ Changes in suspensions of human mononuclear phagocytes stimulated with f-Met-Leu-Phe. Monocytes cultured for 3 d in the presence (*A, C*) or absence (*B, D*) of 60 U/ml rIFNγ were loaded with quin-2 and then stimulated (*arrowheads*) with 10 nM (*A, B*) or 1 nM (*C, D*) f-Met-Leu-Phe. Tracings from a single monocyte preparation are shown. Two additional monocyte preparations each measured in duplicate gave similar results.

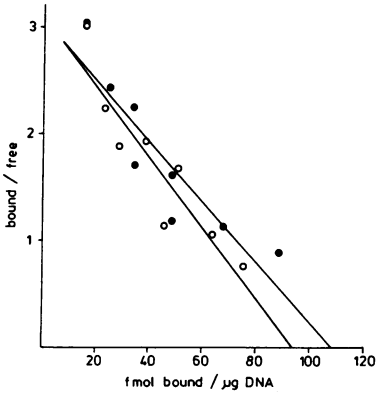

Figure 9. Scatchard analysis of [^{3}H]phorbol dibutyrate binding to control and IFNγ-treated mononuclear phagocytes. Monocytes were cultured for 3 d in the presence or absence of 60 U/ml rIFNγ and than incubated with different concentrations of [^{3}H]PDBu. The values obtained by linear regression analysis were K_D of 35.8 nM and maximum binding sites: 110 fmol/μg DNA for IFNγ-treated cells (●), and K_D = 30.7 nM and maximum binding sites: 95 fmol/μg DNA for control cells (○).

Effects of IFNγ on $[Ca^{2+}]_i$ and protein kinase C. The possibility was considered that IFNγ could influence agonist-dependent $[Ca^{2+}]_i$ changes or the cellular level of protein kinase C. Tracings of $[Ca^{2+}]_i$-associated fluorescence increase induced by f-Met-Leu-Phe are shown in Fig. 8. A rapid rise in $[Ca^{2+}]_i$ was observed with half maximum (1 nM) and saturating (10 nM) concentrations of f-Met-Leu-Phe in IFNγ-treated and control cells. In three different experiments, IFNγ had no appreciable effect on the change induced by 10 nM f-Met-Leu-Phe (Fig. 8, *A* and *B*). At the lower agonist concentration, however, the rate of the $[Ca^{2+}]_i$ rise was consistently somewhat slower (Fig. 8 *C* and *D*), suggesting a possible decrease in the signal transduction efficiency.

The levels of protein kinase C were measured by phorbol ester binding using PDBu. Scatchard analysis were made from four different donors measured in duplicate. Treatment of the mononuclear phagocytes with IFNγ did not affect the affinity and the number of the phorbol ester binding sites. The average K_D were 32.4±1.9 and 31.9±7.7 nM for control and IFNγ-treated cells, respectively. The number of binding sites varied with the donors between 95 and 233 fmol/μg DNA but were not changed after treatment with IFNγ. A representative experiment is shown in Fig. 9. These results indicate further that the higher responsiveness of IFNγ-treated macrophages to PMA does not result from an elevation of protein kinase C. In a recent report, Berton et al. (13) showed that treatment with *Corynebacterium parvum* enhanced the capacity of murine peritoneal macrophages to produce superoxide upon stimulation with PMA, but did not alter the level of protein kinase C.

Discussion

The mechanism of the enhanced PMA-dependent hydrogen peroxide formation in mononuclear phagocytes treated with IFNγ (1) is still incompletely understood. It was shown that the respiratory burst oxidase of IFNγ-treated, PMA-stimulated human macrophages has a lower K_m for NADPH than that of control cells (10). A higher NADPH affinity was also reported for the respiratory burst oxidase of murine peritoneal macrophages activated by infection (11) or by the intraperitoneal administration of various irritants (11–13). In all these studies

the effect of activation in vivo or IFNγ treatment in vitro was assessed using PMA as the stimulus (1, 11–13, 26). Acting on protein kinase C (27, 28), PMA induces a long-lasting respiratory burst generally measured by the amount of hydrogen peroxide accumulating in the culture medium. By contrast stimuli like f-Met-Leu-Phe or PAF, which act via surface receptors, elicit transient responses (29–31) that are more difficult to quantify, and indeed little information is available on the influence of macrophage activation on agonist-mediated respiratory burst.

We have now cultured human monocytes in Teflon bags and have studied the effects of IFNγ on their response to receptor agonists and PMA. Using single-cell suspensions, we have determined the rate of superoxide or hydrogen peroxide production, as a measure of NADPH-oxidase activity. IFNγ treatment increased this rate two- to threefold when PMA was used as stimulus, which is in agreement with the enhanced production of hydrogen peroxide reported earlier (1, 6). Surprisingly, however, IFNγ failed to enhance superoxide formation in response to various concentrations of f-Met-Leu-Phe alone or in combination with PAF. In fact, the rate of superoxide formation obtained in control cells with a maximum effective concentration of f-Met-Leu-Phe was not exceeded under other stimulatory conditions. These results show that stimulation of the mononuclear phagocytes with appropriate (i.e., presumably saturating) concentrations of a receptor agonist results, with or without IFNγ treatment, in a full activation of the NADPH-oxidase and consequent maximum flux of electrons from NADPH to oxygen. A much lower activity is observed when control macrophages are stimulated with maximum effective concentrations of PMA. This response is markedly enhanced by IFNγ, but not above the maximum electron flux obtained with f-Met-Leu-Phe, which appears to correspond to the maximum activity of the NADPH-oxidase in intact cells. Our measurements failed to confirm the effect of IFNγ on the affinity of the PMA-stimulated oxidase for its substrate, NADPH, as reported for human (10) and murine macrophages (11–13). Our K_m values ranged between 30 and 40 μM and were independent of the stimulus used and of IFNγ treatment. Thus the lower rate of superoxide production in untreated cells stimulated with PMA does not appear to result from a lower affinity of the oxidase for NADPH. The discrepancy with respect to the earlier reports (10–13) may depend on the experimental conditions. In our study, the cells stimulated with either PMA or f-Met-Leu-Phe were lysed in the presence of EGTA, and cytochrome *c* reduction upon NADPH addition was recorded continously thus permitting the reliable estimation of initial velocity even at NADPH concentrations well below K_m. It must also be noted that the only K_m determinations so far reported for human mononuclear phagocytes (10) were performed after a culture period of at least eight days. We did not adopt such conditions, since we could assess the effect of IFNγ on hydrogen peroxide production elicited by PMA by a 3-d treatment of freshly isolated blood monocytes.

IFNγ had no measurable effect on other elements of the respiratory burst response as well. As suggested by high-resolution difference spectroscopy, it does not enhance the content of cytochrome b_{558} or other cytochromes in human mononuclear phagocytes, ruling out the possibility of a quantitative change in the assumed terminal component of the NADPH-oxidase. An analogous situation was found in murine peritoneal macrophages where the levels of b-type cytochromes were not altered by in vivo activation (12, 26). Equally unchanged upon treatment with IFNγ were the content and the phorbol ester affinity of protein kinase C, as shown by our Scatchard analysis, indicating that the higher PMA responsiveness of IFNγ-treated cells does not result from an increased availability or activity of protein kinase C. Analogous observations were reported for *C. parvum*-activated mouse macrophages (13). Finally, IFNγ did not appreciably affect the rise in $[Ca^{2+}]_i$ upon f-Met-Leu-Phe stimulation, suggesting that the activation of human mononuclear phagocytes has little influence on the process of transduction of agonist signals. Experiments with low concentrations of f-Met-Leu-Phe showed that if anything IFNγ tends to decrease the responsiveness of the cells to receptor agonists as suggested by a diminished superoxide production and a somewhat slower rising of $[Ca^{2+}]_i$.

This study fully confirms the original observation that IFNγ increases the respiratory burst activity of human macrophages exposed to PMA (1). It shows, however, that IFNγ does not enhance the physiological (and more efficient) response to receptor agonists, and suggests that its effect is restricted to the respiratory burst induced by PMA. Since IFNγ also failed to influence appreciably agonist-induced $[Ca^{2+}]_i$ changes and the cellular levels of cytochrome b_{558} and protein kinase C, the notion that increased NADPH-oxidase activity is an expression of macrophage activation (9, 32) does not appear to apply to human mononuclear phagocytes. Recent kinetic studies on human neutrophils (33) and human monocytes (manuscript in preparation) have shown that f-Met-Leu-Phe initiates the respiratory burst much more rapidly than PMA even at maximum-effective concentrations. It is conceivable that IFNγ enhances the responsiveness of mononuclear phagocytes to PMA by influencing a still unknown intermediate between protein kinase C and the NADPH-oxidase, that may not be involved in the superoxide formation induced by receptor agonist.

These considerations do not question the macrophage-activating properties of IFNγ or the cidal effect of NADPH-oxidase products. Higher expression of class II antigens, Fc and complement receptors, and possibly other surface determinants has been amply documented as a correlate of the acquired capacity of IFNγ-treated macrophages to kill intracellular parasites and tumor cells (see 34–36 for review). Enhanced adherence and phagocytic activity consequent to the remodeling of the macrophage surface membrane can result in stronger respiratory burst responses without actual changes of the NADPH-oxidase and its mechanism of activation. Finally, the reported antiparasitic (37, 38) and antitumor (39) activity by NADPH-oxidase deficient human cells indicate that macrophage activation can be fully dissociated from enhanced superoxide or hydrogen peroxide production.

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