Reconstitution of Cell-Free NADPH-Oxidase From Human Monocytes and Comparison With Neutrophils

By Marcus Thelen and Marco Baggiolini

A rapid centrifugal elutriation procedure was developed to prepare high amounts of pure human blood monocytes. After disruption by nitrogen cavitation, a cytosol and a membrane fraction were obtained by sucrose-Percoll density centrifugation. The plasma membrane fraction contained cytochrome b_{558} and was free of microsomal or mitochondrial cytochromes as determined by low-temperature absorbance spectroscopy. Cell-free NADPH-oxidase activity from monocytes and neutrophils was reconstituted with cytosol and membranes in the presence of sodium dodecyl sulphate. By comparison with neutrophils, the cell-free NADPH-oxidase from monocytes showed a two- to threefold lower specific activity. The NADPH-oxidase was reconstituted with neutrophil membranes and monocyte cytosol, and vice versa. The K_m for NADPH was always lower when monocyte cytosol was used. These experiments indicate that the membrane-bound components of the NADPH-oxidase from neutrophils and monocyte are similar and that levels of NADPH-oxidase components in the cytosols differ. Interferon γ-treatment of the monocytes had no effect on the specific activity of the cell-free NADPH-oxidase.

EXPOSURE OF monocytes to appropriate stimuli results in a large burst of nonmitochondrial respiration in which molecular oxygen is converted to superoxide at the expense of intracellular NADPH.[1] As a consequence of stimulation, the enzyme complex that produces superoxide, the NADPH-oxidase, is converted from an inactive to an active form.[2]

Activation of the NADPH-oxidase in a cell-free system requires appropriate combinations of soluble and plasma membrane-associated proteins and unsaturated fatty acids or sodium dodecyl sulfate (SDS).[3-6] A flavoprotein[2,7,8] and a cytochrome b_{558} with a low midpoint potential of −245 mV[9,10] have been identified as the membrane site where superoxide is formed. Up to four components have been partially characterized in the cytosol.[11-13] During oxidase activation in the presence of SDS or arachidonate, the cytosolic factors apparently become associated with the membrane,[14] and in recent reports it has been suggested that a binding site for NADPH is located in the cytosol.[15,16] The enhancement of the activity by GTP-γ-S and sodium fluoride implies the involvement of a GTP-binding protein in the activation process,[17,18] while the participation of protein kinase C in the cell-free activation of the NADPH-oxidase is controversial.[19-23]

We have studied the reconstitution of the NADPH-oxidase activity of human monocytes in comparison with that of human neutrophils. The present results show that both cells have similar membrane-bound oxidase components, while the cytosolic components from monocytes are less efficient than those from neutrophils in reconstituting the NADPH-oxidase activity.

MATERIALS AND METHODS

Materials. Lympho-paque was obtained from Nyegaard (Oslo, Norway); bovine serum albumin (BSA) from Fluka (Buchs, Switzerland); Percoll from Pharmacia (Glattbrug, Switzerland); cytochrome *c* (type III), heparin, and phorbol 12-myristate 13-acetate (PMA) from Sigma (St Louis, MO); RPMI-1640 and Dulbecco's modified Eagle's medium from GIBCO (Basel, Switzerland). Recombinant interferon γ (IFNγ) (RO 41-3503, 10^8 U/mg) was provided from Hoffmann-La Roche Ltd (Basel, Switzerland); superoxide dismutase (SOD) was kindly provided by Dr L. Flohè (Grünenthal AG, Aachen-Stolberg, FRG). All other chemicals were of analytical grade.

Buffers. Three buffered solutions were used: phosphate-buffered saline (PBS), 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4; buffer A, 100 mmol/L KCl, 10 mmol/L K^+-Pipes, 3.5 mmol/L $MgCl_2$, 3 mmol/L NaCl, pH 7.3; buffer B, 66 mmol/L KCl, 17 mmol/L Na_2HPO_4 6.6 mmol/L K^+-Pipes, 5.8 mmol/L $MgCl_2$, 2 mmol/L NaCl, pH 7.4.

Large-scale purification of human monocytes by centrifugal elutriation. High numbers of monocytes were prepared from donor blood by centrifugal elutriation. Mononuclear cells were separated from buffy coats of single blood units stored at 4 to 10°C for up to 20 hours (Swiss Red Cross Laboratory) by centrifugation through a 0.8 mmol/L sodium citrate-containing Lympho-paque gradient.[24] The cells obtained (0.5 to 1 × 10^9) were diluted in PBS containing 0.5 U/mL of heparin, pelleted (300 × *g* for 10 minutes at 4°C), washed twice with PBS, 0.5 mmol/L EDTA, 5% BSA (250 × *g* for 10 minutes at 4°C) to remove residual platelets, resuspended in 5 mL PBS, 0.27 mmol/L EDTA, 1% BSA. Elutriation was performed at 10°C in the latter medium by a modification of the procedure described by Clemetson et al[25] using a JE-6 rotor in a J-6M centrifuge (Beckman Instruments, CA) and stepwise reducing the centrifugal force and increasing the medium flow. Monocytes from several elutriations (approximately 10^9 cells) were combined and resuspended (10^7 cells/mL) in PBS. On average, the monocyte preparations obtained by this method were 90% pure as assessed by Giemsa staining and peroxidase histochemistry.

Neutrophils were prepared from donor buffy coats as described previously.[24]

Membranes and cytosol fractions from monocytes and neutrophils were prepared at 0°C. EGTA at the final concentration of 1 mmol/L was added to the purified monocytes. The cells were pelleted (250 × *g* for 10 minutes), resuspended in PBS (10^8 cells/mL), and incubated with 2.5 mmol/L diisopropyl fluorophosphate.[26] They were then diluted with an equal volume of PBS containing 2 mmol/L

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EGTA, washed with PBS (250 × *g* for 10 minutes), resuspended in buffer A containing 1 mmol/L adenosine triphosphate (ATP) (7.5×10^7 cells/mL), and disrupted by nitrogen cavitation at 20 atm for 20 minutes. The EGTA concentration in the homogenate was adjusted to 1.25 mmol/L, and a postnuclear supernatant was prepared by centrifugation at 500 × *g* for 10 minutes. Up to 10 mL of the postnuclear supernatant were layered onto a two-step density gradient consisting of 14 mL of 12% sucrose, 25% isotonic Percoll,[27] 1 mmol/L ATP, and 1.25 mmol/L EGTA in buffer A and 14 mL of 90% Percoll in buffer A. After centrifugation (20,000 rpm for 18 minutes at 4°C in a Sorvall SS34 rotor), a fraction corresponding to the volume of the sample and the first visible band (just below the density interface) were collected by aspiration. Both fractions were centrifuged (436,000 × *g* for 15 minutes, TLA-100.2, Beckman). The cytosol collected from the first fraction (7.5×10^7 cell eqiv/mL) contained 1.28 ± 0.48 mg protein/mL (n = 6), the pellets (membranes) from both fractions were resuspended[22] (1.2×10^9 cell eqiv/mL), and the pooled suspension contained 4.5 ± 0.9 mg protein/mL (n = 6). Cytosol and membranes from neutrophils were prepared according to Curnutte et al,[22] the protein concentration of the cytosol (7.5×10^7 cell eqiv/mL) was 1.42 ± 0.3 mg/mL (n = 5) and of the membranes (1.2×10^9 cell eqiv/mL) was 2.59 ± 0.45 mg/mL (n = 4). All subcellular fractions were stored at −70°C for up to 12 month.

Superoxide measurements. NADPH-oxidase activity was measured at 25°C in an HP 8451A diode-array spectrophotometer as described by Thelen et al.[28] Stock membranes from monocytes and neutrophils containing 320 ± 50 pmol cytochrome b_{558} per milligram of protein were diluted 6.25 times with buffer A and briefly sonicated. Approximately 5 to 10 μg of membrane protein was incubated with 50 to 150 μg prewarmed cytosol protein in buffer B containing 85 μmol/L ferricytochrome *c*, 1 mmol/L NaN_3 (final concentrations in 750 μL) for 5 minutes in the presence of 15 mmol/L NaF and 128 μmol/L SDS. Superoxide production was detected after addition of 160 μmol/L NADPH. Superoxide-independent cytochrome *c* reduction was determined from replicate samples to which 30 μg of SOD was added 2 to 10 minutes after activation of the NADPH-oxidase. Maximum rates of superoxide production were calculated from the linear parts of the traces and corrected for SOD insensitive cytochrome *c* reduction.

Spectroscopy. Cytochrome b_{558} content was measured at room temperature in 50 mmol/L sodium phosphate buffer using an extinction coefficient of $\epsilon_{559\text{-}540} = 21.6$ mmol/L^{-1} cm^{-1}.[29] High resolution low temperature spectra were recorded at 77°K.[28]

IFNγ-treatment was performed as described previously.[28] Protein was measured with the bicinchoninic acid assay reagent (Pierce) according to the manufacturer's instructions.

RESULTS

Large-scale preparation of human blood monocytes. An abbreviated elutriation procedure adopted to prepare high numbers of cells yielded purified monocytes from single buffy coats in 30 to 40 minutes. The average yield was 130 ± 37 × 10^6 monocytes per buffy coat (820 ± 200 mononuclear cells, n = 15), and the purity ranged between 86% and 94%. Contaminating cells were mainly basophil granulocytes and lymphocytes.

Plasma membrane preparation. Sucrose-Percoll density gradients centrifugation yielded membrane fragments with high NADPH-oxidase activity. Low-temperature spectroscopy of these membranes showed the typical reduced minus oxidized spectrum of the low-potential cytochrome b_{558} with an absorbance maximum at 558 nm and a shoulder at 556 nm (Fig 1).[30] No other hemoproteins were detectable, indicating that the preparation was virtually free of microsomal and mitochondrial contamination. A similar degree of spectroscopic purity was obtained with neutrophil membrane preparations.

Cell-free activation of the NADPH-oxidase. Mixing of monocyte membranes and cytosol in the presence of SDS reconstituted the NADPH-dependent, SOD-sensitive superoxide production typical of the respiratory burst oxidase. In the absence of either cytosol or plasma membrane, no activity was obtained. On centrifugation of the active mixture (436,000 × *g* for 10 minutes) about 80% of the NADPH-dependent superoxide production was recovered in the pellet and only minor losses of activity were observed on washing, provided that 100 to 130 μmol/L SDS was present. By contrast, the supernatant (cytosol) was devoid of NADPH-oxidase activity and could not be used to further reconsitute the activity on addition of fresh membranes (data not shown).

In confirmation of previous work,[22] a similar cell-free NADPH-oxidase preparation was obtained from membrane and cytosol fractions of neutrophils. As shown in Fig 2A, the NADPH-oxidases from monocytes and neutrophils showed equal dependence on SDS, although the neutrophil enzyme was more active. We tested for the possible involvement of a GTP-binding protein in the activation of the NADPH-oxidase using sodium fluoride, that in the presence of GDP can activate GTP-binding proteins.[18,31] Sodium fluoride enhanced the activity of both cell-free preparations in a concentration-dependent manner (Fig 2B).

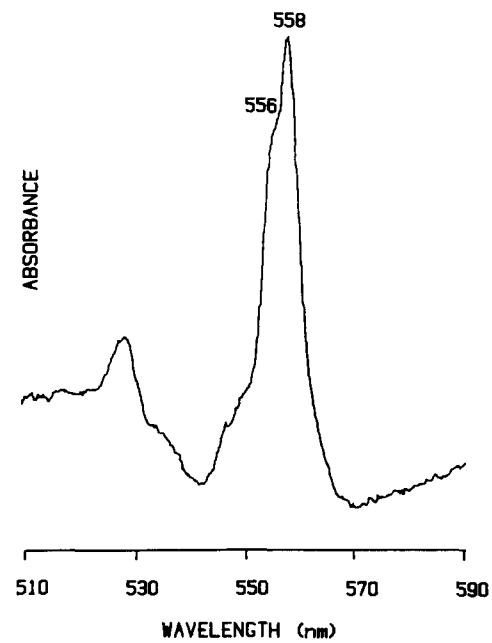

Fig 1. Low-temperature reduced minus oxidized absorbance difference spectrum of monocyte plasma membranes. Plasma membranes from approximately 6 × 10^7 cells were suspended in 250 μL buffer A. Oxidized and dithionite-reduced low-temperature (77°K) spectra were recorded successively from 510 nm to 590 nm and then subtracted from each other.

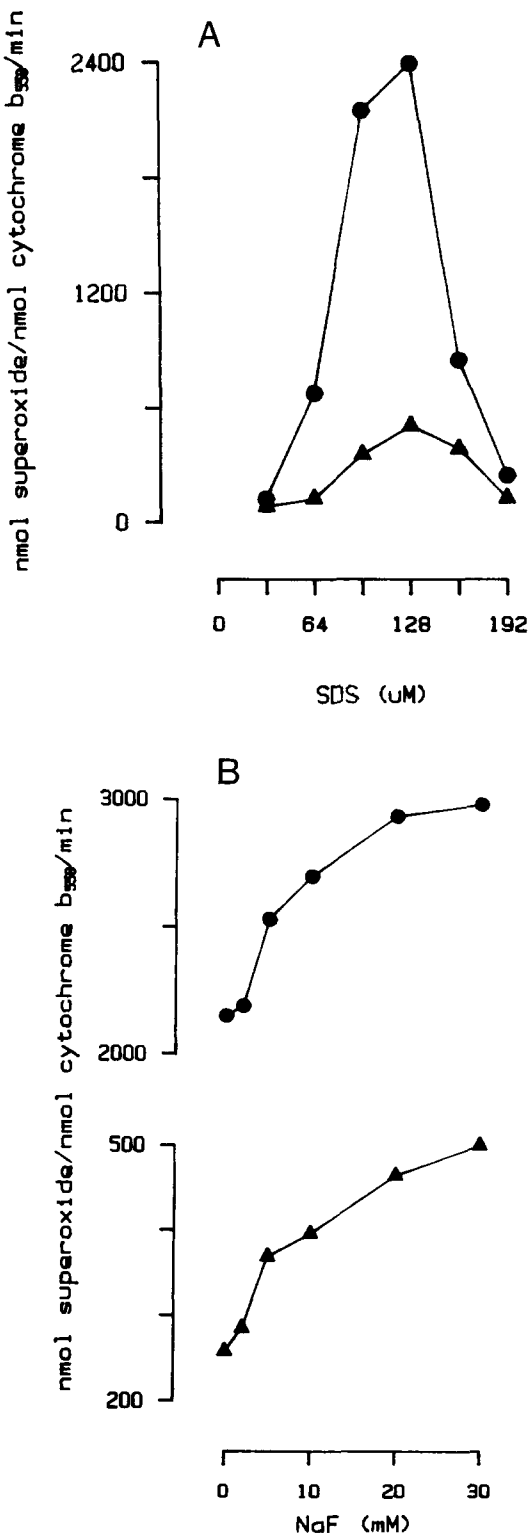

Fig 2. Effect of SDS and sodium fluoride on the NADPH-oxidase activation. Membranes and cytosol from monocytes (▲) or neutrophils (●) were incubated at 25°C for 5 minutes (A) in the presence of 15 mmol/L sodium fluoride and increasing concentrations of SDS, and (B) in the presence of 128 μmol/L SDS and increasing concentrations of sodium fluoride. Superoxide production was then initiated by the addition of 160 μmol/L NADPH. The results are representative for five similar experiments.

Specific activity of the cell-free NADPH-oxidase. Table 1 shows that the specific activity of the cell-free NADPH-oxidase from monocytes was two- to threefold lower when compared with neutrophils. In agreement with previous observations,[32] maximum superoxide production was obtained using 7 to 10 cell equivalents of cytosol per cell equivalent of membrane.

Effect of cytosol components. The NADPH-oxidase activity was reconstituted with membranes from monocytes or neutrophils and different amounts of cytosol from either cell type. Assuming that cytochrome b_{558} is an essential and stoichiometric component of the NADPH-oxidase, the results obtained with membranes from monocytes and neutrophils were normalized according to the relative content of the cytochrome b_{558} of each preparation. As shown in Fig 3, the NADPH-oxidase activity increased depending on the amount of cytosolic protein. At a ratio of 120 to 150 mg cytosol protein per nmol cytochrome b_{558} (approximately 6 to 8 cell equivalents cytosol per cell equivalent membrane), superoxide production approached a maximum with either source of cytosol. Reconstitution with monocyte cytosol resulted in markedly lower activity than reconstitution with neutrophil cytosol. On the other hand, the activity was independent of the source of membranes, suggesting that the membrane-associated part of the superoxide producing enzyme is similar in both types of cells, while some difference must exist among the cytosolic components from monocytes and neutrophils. A further indication for a difference in the cytosol components was given by the K_m for NADPH as determined from the maximum rate of superoxide production. As shown in Table 2, the K_m values obtained in the presence of monocyte cytosol were always lower than those obtained in the presence of neutrophil cytosol.

No effect of IFNγ. In human monocytes the respiratory burst response to phorbol esters is enhanced two- to threefold by exposure to 100 U/mL IFNγ.[33,34] Because IFNγ did not enhance the level of cytochrome b_{558},[28] we monitored possible changes in the cytosol components. The NADPH-oxidase activity was reconstituted with cytosol and membranes from IFNγ-treated and control monocytes. As indicated in Fig 4, at all cytosol to membrane ratios tested the activity of the reconstituted NADPH-oxidase was unaffected by treatment of the cells with IFNγ.

DISCUSSION

The active NADPH-oxidase of phagocytes is believed to consist of several components. A membrane bound flavoprotein[2,7,8] and a cytochrome b_{558}[9,10] together with at least four cytosolic proteins including an NADPH-binding site,[15,16] have been identified. Some of the membrane components have been characterized,[35-38] and recently several cytosolic components have been partially purified[11-13,39] or functionally cloned from an expression library.[40]

The successful reconstitution of the cell-free NADPH-oxidase from human monocytes and the comparison with the oxidase of human neutrophils was achieved by a number of methodologic innovations. The fast elutriation procedure was essential for obtaining sufficient amounts of cytosol and membrane fractions from monocytes. Similarly critical was

Table 1. NADPH-Oxidase Activity as a Function of Cytosol Concentration

Ratio: Cytosol/Membrane (in cell equivalents)	Activity: nmol O_2^-/min/10^7 Cell Equivalents of Membrane			
	Monocytes	(n)	Neutrophils	(n)
2.0	—		5.4 ± 1.8	6
3.0	—		13.7 ± 3.0	4
3.7	4.7 ± 1.1	7	16.4 ± 4.4	5
4.2	5.7 ± 1.6	6	—	
5.5	7.9 ± 1.7	8	21.0 ± 5.7	7
7.0	10.8 ± 1.8	7	22.7 ± 5.7	7
9.0	11.2 ± 1.6	7	—	

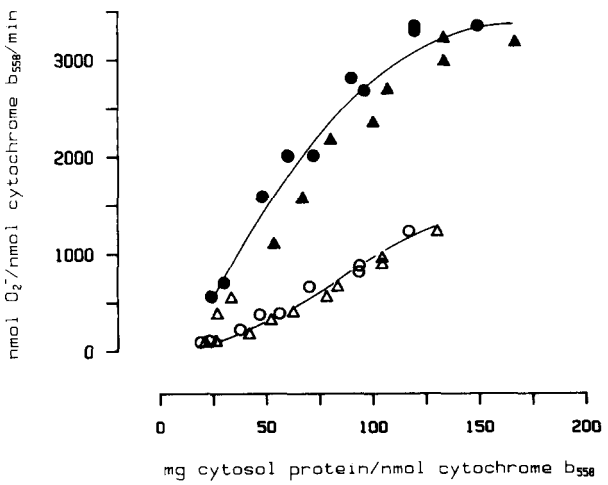

Fig 3. Effect of cytosol on NADPH-oxidase activity. Fixed amounts of membranes from monocytes (△,▲) and neutrophils (○,●) were incubated for 5 minutes with increasing amounts of cytosol from monocytes (△,○) or neutrophils (▲,●) in the presence of 128 μmol/L SDS and 15 mmol/L sodium fluoride. Superoxide production was then initiated by addition of 160 μmol/L NADPH. Monocyte membrane samples contained 1.76 pmol cytochrome b_{558} (4.9 μg protein) and neutrophil membrane samples contained 1.52 pmol cytochrome b_{558} (5.3 μg protein). Means of duplicate measurements from 1 of 3 similar experiments.

the preparation of monocyte membranes by density gradient centrifugation in sucrose-Percoll rather than in Percoll alone, as commonly used for neutrophils,[14,22,27] which was found to bind membrane fragments and thus lower recovery. Finally, low-temperature absorbance spectra showed that the purified membranes (from monocytes and neutrophils) contained only cytochrome b_{558}, therefore it was possible to compare different oxidase preparations on the basis of their *b*-cytochrome content, as determined spectroscopically at room temperature.

The specific activity of the NADPH-oxidase reconstituted from monocytes was markedly lower than that of neutrophils. Because this study showed that the NADPH-oxidase can be reconstituted using the cytosolic components from monocytes and the membrane-associated components from neutrophils and vice versa, the difference in the specific activities could be attributed to the cytosol components of the monocyte enzyme. In fact, membranes from either cell sources yielded equally low NADPH-oxidase activities when reconstituted with monocyte cytosol and equally high activities with neutrophil cytosol. It does not appear that the lower reconstituting capacity of monocyte cytosol was due to generally lower levels of cytosolic factors since optimum NADPH-oxidase activity was obtained for both cell types at similar cytosol to cytochrome b_{558} ratios. However, considering the number of distinct cytosolic proteins of the NADPH-

Table 2. K_m for NADPH of Cell-Free NADPH-Oxidase From Monocytes and Neutrophils

Experiment No.	Source of Cytosol and Membranes			
	Nc + Nm	Nc + Mm	Mc + Nm	Mc + Mm
1	40.0	33.8	21.8	21.0
2	29.8	33.5	25.8	21.0
3	36.2	25.0	19.1	
4	83.0	82.2	55.5	55.5
5	83.0	82.2	55.5	55.5
6	58.8	43.8	30.5	30.1
7	58.3	48.3	38.8	19.7
8		47.0	26.0	35.7
Mean	55.6 ± 20	49.5 ± 20.3	34.1 ± 13.5	36.0 ± 13.6

K_m values are in micromolar NADPH. Determinations were made with six different membrane and cytosol preparations from neutrophils and monocytes using Eadie-Hoffstee plots. The NADPH concentration range was from 20 to 200 μmol/L. The cytosol to membrane ratio was 120 to 150 mg protein/nmol cytochrome b_{558} and was similar for the K_m determinations in each experiment. The average K_m for NADPH using neutrophilic cytosol with membranes from either cell type was 52.3 ± 20.4 μmol/L (n = 15), whereas the average K_m for NADPH for monocytic cytosol tested with both sources of membranes was 35.0 ± 13.6 μmol/L (n = 15).

Abbreviations: N, neutrophil; M, monocyte; c, cytosol; m, membrane.

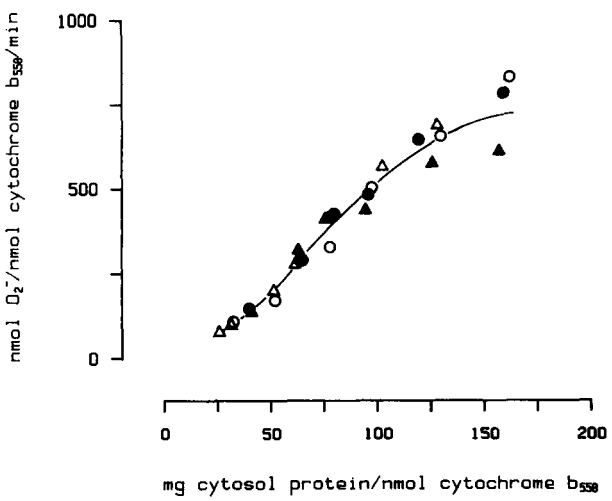

Fig 4. Effect of IFNγ pretreatment on the cell-free NADPH-oxidase activity reconstituted from monocyte fractions. Membranes and cytosol were prepared from monocytes cultured for 3 days in the presence or absence of 100 U/mL recombinant IFNγ. Cell-free NADPH-oxidase activity was reconsituted with membranes from control cells plus cytosol from control (○) or IFNγ-treated cells (●), and with membranes from IFNγ-treated cells plus cytosol from control (△) or IFNγ-treated cells (▲). Means of duplicate measurements from 1 of 3 similar experiments.

oxidase,[13] it is conceivable that monocytes express less or even lack some of the factors while other cytosolic components are at similar levels as in neutrophils. In assent with this view it has been reported that the cytosol from certain patients with autosomal chronic granulomatous disease does not reconstitute the NADPH-oxidase in vitro, but does support the formation of an ordinary oxidase in the presence of normal cytosol. This reconstituted oxidase showed a considerably lower V_{max}, but had a similar K_m for NADPH as the oxidase from control neutrophils.[32] The K_m values for NADPH presented in our study further indicate differences at a functional level. A cytosolic binding site for NADPH has been reported recently.[15,16] The slight difference in the potency of sodium fluoride in increasing the NADPH-oxidase activity from monocytes and neutrophils suggests different levels of GTP-binding proteins or of GDP in the cytosols. The effect of sodium fluoride is consistent with the activation of G-proteins. However, the present data do not exclude the possibility that fluoride acts through other mechanisms, eg, chaotropic or regulatory effects.

Because it is known that activation of human monocytes with IFNγ enhances their respiratory burst capacity,[33,34] we looked for possible effects of IFNγ treatment on the reconstituting capacity of monocyte cytosol and membranes. No effect was observed on either the soluble or the membrane component of the NADPH-oxidase, and there was no change of the cytosol to cytochrome b_{558} ratios for maximum activity. The results confirm previous experiments showing that receptor agonists stimulate maximum superoxide formation in untreated cells and that IFNγ-enhanced, PMA-dependent NADPH-oxidase activity did not exceed the rate of agonist-stimulated superoxide formation. Thus, IFNγ-treatment did not affect the level of the NADPH-oxidase in monocytes.[28] It has been reported that IFNγ-treatment of patients with chronic granulomatous disease enhances their level of cytochrome b_{558} and/or cytosolic factors restoring defective phagocytic function.[41-43] It is conceivable that IFNγ treatment is more effective in cells with defective NADPH-oxidase than in normal cells.

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