Volume 268, number 1, 269-273 FEBS 08672 July 1990

NADPH:O_2 oxidoreductase of human eosinophils in the cell-free system

Ben G.J.M. Bolscher, Leo Koenderman, Anton T.J. Tool, Petra M. Stokman and Dirk Roos

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

Received 23 May 1990

The NADPH oxidase of human eosinophils, measured in the cell-free system, shows the same characteristics as the enzyme from human neutrophils. All proteins required for activity of the enzyme are expressed in eosinophils at a higher level than in neutrophils. Eosinophils isolated from patients with chronic granulomatous disease show the same molecular defects as the neutrophils fom these patients.

Eosinophil; NADPH oxidase; Cell-free system

INTRODUCTION

Eosinophilic granulocytes are important cells in the host defense against pathogenic parasites and micro-organisms. When challenged with appropriate stimuli, eosinophils – like neutrophilic granulocytes – produce toxic oxygen metabolites by means of a membrane-bound NADPH oxidase [1,2]. A *b*-type cytochrome (cytochrome b_{558}) is a component of this enzyme. Eosinophils contain a 2–3-fold higher amount of cytochrome b_{558} than neutrophils as determined by absorption spectroscopy [3]. In neutrophils from patients with X-linked cytochrome b_{558}-negative chronic granulomatous disease (Xb^- CGD), this cytochrome is absent [4].

Recently, it was found that the neutrophil NADPH oxidase can be activated in a cell-free system by addition of sodium dodecyl sulfate or arachidonic acid [5–7]. This cell-free system requires both a plasma membrane and a cytosolic fraction [6]. Two to four cytosolic components from neutrophils have been described that act in concert [8–10]. Two of these components have been characterized as a phosphoprotein of M_r 47 000 (pp47) [8,9,11] and a protein of M_r 67 000 (p67) [9,11], respectively. Two forms of autosomal cytochrome b_{558}-positive chronic granulomatous disease (AB^+ CGD) exist that lack either protein in the neutrophils [12].

Correspondence address: D. Roos, c/o Publication Secretariat, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, PO Box 9406, 1006 AK Amsterdam, The Netherlands

Abbreviations: CGD,chronic granulomatous disease; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMA, phorbol myristic acid; STZ, serum-treated zymosan; Moab, monoclonal antibody; Xb^-, X-linked cytochrome b_{558}-negative, Ab^+, autosomal cytochrome b_{588}-positive

In this report we compared the NADPH oxidase of human eosinophils with that of human neutrophils. Eosinophil NADPH oxidase too can be activated in a cell-free system with SDS. In addition, cytosol from eosinophils can elicit activity in neutrophil plasma membranes and vice versa. Thus eosinophils express at least twice as much of the proteins required for NADPH oxidase activity as do neutrophils.

MATERIALS AND METHODS

2.1. *Materials*

Phenylmethylsulfonyl fluoride (PMSF), acetyl-L-leucyl-L-leucyl-L-arginine (leupeptin), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (hepes), platelet-activating factor (PAF; L-α-phosphatidylcholine, β-acetyl, γ-*O*-hexadecyl) and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were from Sigma Co., St Louis, MO, USA. Keyhole Limpet Hemocyanin (KLH) was obtained from Calbiochem, San Diego, CA, USA. Guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S) and NADPH were purchased from Boehringer, Mannheim, FRG. Phorbol myristic acetate (PMA) was from Consolidated Midland Corp., Katonah, NY, USA. Serum-treated zymosan was prepared as described before [12]. Reagents and molecular-weight markers for SDS-polyacrylamide gelelctrophoresis (SDS-PAAGE) were from Bio-Rad Laboratories, Richmond, CA, USA.

2.2. *Assays for marker enzymes and proteins*

For measurement of eosinophil peroxidase activity, samples were diluted 1:1 with 1% (w/v) cetyltrimethylammonium bromide (CETAB) and shaken vigorously. Samples were centrifuged during 10 min at 10 000 × g and 25 and 50 μl of the clear supernatant were tested for EPO activity. The assay mixture contained 50 mM potassium phosphate, 50 μM KBr, 100 μM H_2O_2 and 40 μM monochlorodimedon, pH 4.0. The reaction was started by the addition of enzyme, and the decrease in absorbance at 280 nm was recorded.

Activities of alkaline phosphatase and lactate dehydrogenase were measured as described by Borregaard et al. [14]. Protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA).

2.3. *Isolation and fractionation of granulocytes*

Eosinophils were isolated at 3 occasions from the buffy coats of 500

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

ml of healthy-donor blood, according to Koenderman et al. [15]. The purity of the isolated eosinophils was 99% in all cases. Neutrophils were isolated as previously described [16]. At one occasion eosinophils were isolated from CGD patients. One patient was classified as X-linked cytochrome b_{558}-negative (Xb^-) and the other as autosomal cytochrome-b_{558}-positive (AB^+) [16]. From the Xb^- CGD patient 10^7 eosinophils were purified from 50 ml of blood, and from the Ab^+ CGD patient 3×10^6 from 25 ml of blood.

For fractionation of normal eosinophils, the cells were suspended in 0.34 M sucrose, 10 mM Hepes, 1 mM EGTA, 1 mM PMSF, 100 μM leupeptin in PBS, pH 7.0, at a concentration of 2×10^7 cells/ml. The cells were disrupted by sonication for three 15-s intervals at 21 kHz frequency and 8 μm peak-to-peak amplitude. The sonicate was centrifuged during 10 min at $800 \times g$ to remove nuclei and unbroken cells. Aliquots (1.3 ml) of the supernatant were layered on top of discontinuous gradients of 1.5 ml 40% (w/v) sucrose and 1 ml 15% sucrose (w/v). The gradient was centrifuged during 60 min at $100\,000 \times g$. The application zone (referred to as cytosol), the interface between the 15%/40% sucrose layer (referred to as plasma membranes) and the fraction on the bottom of the tube (referred to as granules) were harvested and stored at -70°C until further use.

Neutrophils were fractionated in a similar way, except that a 15%/50% (w/v) sucrose gradient was used. The interface of the sucrose layers contained essentially all the plasma membranes and specific granules.

2.4. *Immunoblotting with monoclonal antibodies 449 and 48*

SDS-PAGE followed by immunoblotting with monoclonal (Moab) 449 and Moab 48, directed against the α-chain and β-chain of cytochrome b_{558}, respectively, was performed as described by Verhoeven et al. [18].

2.5. *Immunoblot with polyclonal rabbit antiserum B-1*

Twenty-five μg of cytosol protein were separated by SDS PAGE on 9% slab gels, and blotted onto nitrocellulose sheets. Development of the sheets with polyclonal antiserum B-1 was performed as described in [11].

4,2.6. *Production of polyclonal antiserum PS-47-23*

A peptide corresponding to the amino acids 214–223 of the pp47 NADPH oxidase component [19] was synthesized with an extra N-terminal cysteine. Ten mg of peptide was linked to 30 mg of KLH with SPDP as a linker. The conjugate (250 μg) was injected intramuscular in a New Zealand White rabbit, followed by two booster injections. On an immunoblot with neutrophil cytosol the antiserum reacted specifically with a protein of 47 kDa. No reaction was observed with cytosol from neutrophils derived from Ab^+ CGD patients.

2.7. *Measurement of NADPH oxidase activity in the cell-free system*

NADPH oxidase activity was measured as the rate of oxygen consumption with a Clark oxygen electrode at 27°C. The assay mixture (0.25 ml) was composed of oxidase buffer (10 mM Hepes, 10 mM potassium phosphate, 0.17 M sucrose, 25 mM NaCl, 0.5 mM EGTA, 1 mM $MgCl_2$, 2 mM sodium azide, 10 μM GTP-γS; pH 7.0, 5 μg of plasma membrane protein and 20 μg of cytosol protein. The reaction was initiated by the addition of 10 μl of SDS to a final concentration of 100 μM, as described by Bromberg and Pick [6]. The reaction vessel was closed, 3 min later NADPH was added to a final concentration of 250 μM, and the NADPH-dependent oxygen consumption was determined.

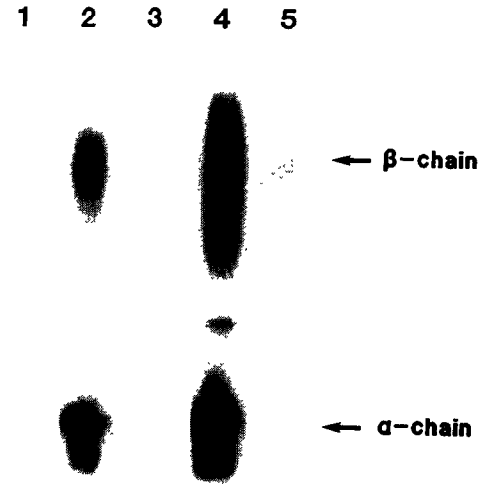

Fig. 1. Western Blot of neutrophil and eosinophil cell fractions with MoAb 48 and MoAb 449. Fractions corresponding to 2×10^6 cell equivalents were run on a 5–15% SDS polyacrylamide gel, and Western blotting was performed as described in section 2. (lane 1) Neutrophil cytosol; (lane 2) neutrophil plasma membranes plus neutrophil specific granules (this contains all the neutrophil cytochrome b_{558}); (lane 3) eosinophil cytosol; (lane 4) eosinophil plasma membranes; and (lane 5) eosinophil granules.

2.8. *Measurement of oxygen consumption by intact eosinophils*

Oxygen consumption was measured at 37°C with a Clark oxygen electrode. The eosinophils were suspended at a concentration of 2×10^6 cells per ml in 133 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM Hepes, 1.2 mM potassium phosphate, 5.5 mM glucose, and 0.5% (w/v) human albumin, pH 7.4. After 15 min of preincubation the stimulus was added and the oxygen consumption was recorded for 5 min. The results are given as the maximum rate of oxygen consumption during this interval.

RESULTS AND DISCUSSION

3.1. *Fractionation of eosinophils*

Table I shows the result of a fractionation of human eosinophils. Little cross-contamination was observed between the three major fractions. Especially, the

Table I

Fractionation of human eosinophils

Fraction	Protein	Eosinophil peroxidase (% of total)	Lactate dehydrogenase
cytosol	51.8 ± 3.2	0	98.4 ± 1.1
plasma membranes	8.0 ± 2.2	0.5 ± 0.2	0.8 ± 0.2
granules	40.2 ±3.0	99.5 ± 0.7	0.8 ± 0.1

At three occasions eosinophils were isolated from peripheral blood and fractionated as described in section 2. The amount of eosinophils was 45×10^6, 38×10^6 and 52×10^6. The results are the means ± SE.

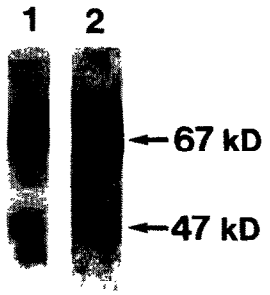

Fig. 2. Immunoblot with antiserum B-1. Samples containing 1.5×10^6 cell equivalents were run on a 5–15% SDS polyacrylamide gel, and Western blotting with antiserum B-1 was performed as described in section 2. (lane 1) neutrophils; (lane 2) eosinophils.

plasma membranes are essentially free of granule and cytosol markers. When we attempted to measure alkaline phosphatase as a plasma membrane marker we found neither activity in any of the fractions, nor in intact eosinophils. Because our eosinophils are essentially devoid of neutrophils, the activity of alkaline phosphatase reported in previous studies with eosinophils is most likely due to contaminating neutrophils [20]. The absence of alkaline phosphatase is not due to our methods of isolation, because alkaline phosphatase was also undetectable in eosinophils isolated by a different method [1].

We further characterized the plasma membrane fraction by estimation of the amount of cytochrome b_{558}. It has been reported that eosinophils contain twice as much cytochrome b_{558} as do neutrophils [3]. Fig. 1 shows a Western blot with the monoclonal antibodies Moab 449 and Moab 48, directed against the α and β subunit of cytochrome b_{558}, respectively [18]. The majority of cytochrome b_{558} was found in the eosinophil plasma membrane fraction and little in the granule fraction. This was confirmed by absorption spectroscopy (not shown). Because cytochrome b_{558} in eosinophils is mainly located in small cytoplasmic granules [21], it is likely that the interface between the 15% and 40% sucrose contains these granules as well. In addition, Fig. 1 shows that eosinophils contain both subunits of cytochrome b_{558} in higher quantities than neutrophils.

For activation of the NADPH oxidase in neutrophils both membrane-bound and cytosolic proteins are required [6]. Two cytosolic proteins have been characterized, a 47-kDa phosphoprotein [8,9,12] and a protein of 67 kDa [9,12]. These proteins can be visualized by immunoblotting with a polyclonal rabbit antiserum B-1 [11]. Fig. 2 shows an immunoblot of

Table II

Activity of the NADPH oxidase in the cell-free system

Source of cytosol	Source of membranes	O_2 consumption (nmol/min/mg membrane protein)
neutrophils	neutrophils	412 ± 80
eosinophils	eosinophils	941 ± 125
neutrophils	eosinophils	649 ± 35
eosinophils	neutrophils	696 ± 59
eosinophils	granules from eosinophils	118 ± 18

NADPH oxidase was measured in the cell-free system as described in section 2 with 5 μg of plasma membrane protein and 20 μg of cytosol protein. Under these conditions the assay is rate-limited by both plasma membranes and cytosol. Values are the mean ± SE of 3 experiments.

Table III

Respiratory burst activity of eosinophils from CGD patients

Source of eosinophils	Stimulus	O_2 consumption (nmol/min/10^6 cells)
Xb^--patient	10^{-6} M PAF + 1 mg/ml STZ	0.15
Xb^--patient	200 ng/ml PMA	0.06
Ab^+-patient	10^{-6} M PAF + 1 mg/ml STZ	0.30
Ab^+-patient	200 ng/ml PMA	0.08
Control	10^{-6} M PAF + 1 mg/ml STZ	14.2 ± 1.0 ($n=5$)
Control	200 ng/ml PMA	16.1 ± 2.5 ($n=5$)

Oxygen consumption was measured at 37°C with a Clark oxygen electrode as outlined in section 2. Cells were preincubated during 15 min before the stimulus was added, and the oxygen consumption was recorded for 5 min. PAF was added 2 min before the addition of STZ. The results show the maximum rate of oxygen consumption. For the control studies, the mean ± SE are given.

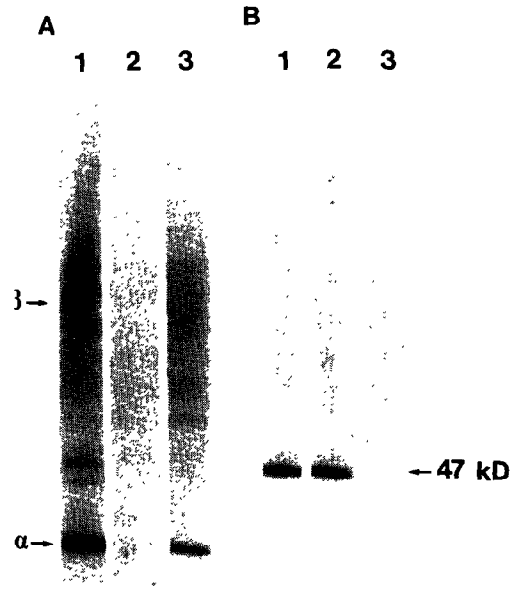

Fig. 3. Immunoblot of eosinophils from CGD patients with MoAb 449 and MoAb 48 and polyclonal antiserum PS-47-23. (A) Detection of the α and β subunit of cytochrome b_{558}. (B) Detection of the 47 kDa protein with antiserum PS-47-23. (Lane 1) 10^6 control eosinophils; (lane 2) 10^6 eosinophils from a patient with Xb^- CGD; (lane 3) 5×10^5 eosinophils from a patient with Ab^+ CGD.

cytosol from eosinophils and neutrophils with antiserum B-1. It is clear that both proteins are present in the cytosol of eosinophils.

3.2. *NADPH oxidase activity of eosinophils in the cell-free system*

Table II shows the activity of the NADPH oxidase in the cell-free system. Under conditions in which activity of the enzyme is rate-limited by both the membrane-bound and the cytosolic components, eosinophils expressed approximately 2.5 times higher activity than neutrophils. When the plasma membrane fraction from eosinophils was replaced by the granule fraction, about 10% of the activity was found. This agrees well with the amount of cytochrome b_{558} found in the granule fraction (Fig. 1). In addition, Table II shows that plasma membranes from eosinophils can be reconstituted with cytosol from neutrophils and vice versa, giving intermediate values of oxygen consumption. These results strongly suggest that eosinophils and neutrophils express the same proteins required for the NADPH oxidase.

3.3. *Eosinophils from patients with chronic granulomatous disease*

To investigate whether eosinophils from CGD patients have the same molecular defect as the neutrophils from these patients, we isolated eosinophilic granulocytes from two patients with chronic granulomatous disease (CGD). One patient was characterized as X-linked cytochrome b_{558}-negative (Xb^-) and the other as autosomal cytochrome b_{558}-positive (Ab^+). Neutrophils from both patients failed to generate a respiratory burst when challenged by appropriate stimuli (not shown). Table III shows that eosinophils isolated from these patients showed no oxygen consumption with either PAF plus opsonized particles or PMA as a stimulus. This indicates that the genetic defect of these patients is manifested in their eosinophilic granulocytes as well. To characterize this further, an immunoblot was performed with eosinophils from both types of CGD patients. Fig. 3 shows that in the eosinophils from an Xb^- patient both subunits of cytochrome b_{558} were absent, whereas in eosinophils from an Ab^+ patient the 47 kDa protein was undetectable.

3.4. *Conclusions*

In this paper we have demonstrated that the NADPH oxidase of eosinophils can be activated in the cell-free system with SDS. In addition, we have shown that eosinophils express the same proteins as neutrophils, and the expression of these proteins in eosinophils is at least twice as high as in neutrophils. Genetic defects of these proteins are manifested both in neutrophils and in eosinophils.

Acknowledgements: We thank Dr Robert A. Clark of the Department of Medicine, University of Iowa, for the immunoblots with antiserum B-1. This study was financially supported by a grant from the Foundation for Medical Research (MEDIGON), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO) (Grant 900-503-091).

REFERENCES

[1] Yazdanbakhsh, M., Eckmann, C.M. and Roos, D. (1985) J. Immunol. 135, 1378–1384.
[2] Yamashita, T., Someya, A. and Hara, E. (1985) Arch. Biochem. Biophys. 241, 447–452.
[3] Segal, A.W., Garcia, R., Goldstone, A.H., Cross, A.R. and Jones, O.T.G. (1981) Biochem. J. 196, 363–267.
[4] Segal, A.W., Jones, O.T.G., Webster, D. and Allison, A.C. (1978) Lancet 2, 446.
[5] Heyneman, R.A. and Vercauteren, R.E. (1984) J. Leuk. Biol. 36, 751–759.
[6] Bromberg, Y. and Pick, E. (1985) J. Biol. Chem. 260, 13539–13545.
[7] Curnutte, J.T. (1985) J. Clin. Invest. 75, 1749–1743.
[8] Bolscher, B.G.J.M., Van Zwieten, R., Kramer, IJ.M., Weening, R.S., Verhoeven, A.J. and Roos, D. (1989) J. Clin. Invest. 83, 757–763.
[9] Nunoi, H.N., Rotrosen, D., Gallin, J.I. and Malech, H.L. (1988) Science 242, 1298–1301.
[10] Curnutte, J.T., Scott, P.J. and Mayo, L.A. (1989) Proc. Natl. Acad. Sci. USA 86, 825–829.
[11] Volpp, B.D., Nauseef, W.M. and Clark, R.A. (1988) Science, 242, 1295–1297.
[12] Clark, R.A., Malech, H.L., Gallin, J.I., Nunoi, H., Vollp, B.D., Pearson, D.W., Nauseef, W.M. and Curnutte, J.T. (1989) N. Engl. J. Med. 321, 647–651.
[13] Goldstein, I.M., Roos, D., Kaplan, H.B. and Weissmann, G. (1975) J. Clin. Invest. 56, 11551–1161.

[14] Borregaard, N., Heiple, J.M., Simons, E.R., Clark, R.A. (1983) J. Cell Biol. 97, 52–61.
[15] Koenderman, L., Kok, P.T.M., Hamelink, M.L., Verhoeven, A.J. and Bruynzeel, P.L.B. (1988) J. Leuk. Biol. 44, 79–86.
[16] Lutter, R., Van Schaik, M.L.J., Van Zwieten, R., Wever, R., Roos, D. and Hamers, M.N. (1985). J. Biol. Chem., 260, 2237–2244.
[17] Hamers, M.N., De Boer, M., Meerhof, L.J., Weening, R.S. and Roos, D. (1984) Nature 307, 553–555.
[18] Verhoeven, A.J., Bolscher, B.G.J.M., Meerhof, L.J. Van Zwieten, R., Keyer, J., Weening, R.S. and Roos, D. (1989) Blood, 73, 1686–1694.
[19] Volpp, B.D., Nauseef, W.M., Donelson, J.E., Moser, D.R. and Clark, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 7195–7199.
[20] Kroegel, C., Yukawa, T., Dent, G., Venge, P., Chung, K.F. and Barnes, P.J. (1989) J. Immunol. 142, 3518–3526.
[21] Ginsel, L.A., Onderwater, J.J.M., Fransen, J.A.M., Verhoeven, A.J. and Roos, D. (1990) Blood (submitted).